ANNALS OF THE NEW YORK ACADEMY OF SCIENCES

VOLUME 66, ART. 2, PAGES 233–414

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NATURAL RESISTANCE TO INFECTIONS

BY

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NEW YORK
PUBLISHED BY THE ACADEMY
October 5, 1956

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(Founded in 1817)

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NATURAL RESISTANCE TO INFECTIONS*

Conference Chairman: WALSH MCDERMOTT
Consulting Editor: J. S. KISER

CONTENTS

* This series of papers is the result of a conference on *Natural Resistance to Infections* by the Section of Biology of The New York Academy of Sciences, March 1 and 2, 1956.

THE NATURE OF THE PROPERDIN SYSTEM AND ITS INTERACTIONS WITH POLYSACCHARIDE COMPLEXES*

By Louis Pillemer

Institute of Pathology, Western Reserve University, Cleveland, Ohio

Natural resistance to infections is a function of many variables, some of which are definitely recognizable and capable of measurement, while others, probably of equal importance, have so far evaded precise scientific treatment. The phagocytes, for instance, may engulf and destroy bacteria. Special agents, such as lysozyme, kill certain bacteria. Many organs, perhaps by means of reticuloendothelial cells, combat infection.

The significance of all of these mechanisms of natural resistance and their interrelations is not fully understood. There are many reasons for this. The obvious importance of acquired immunity and the ease with which antibodies can be studied has resulted in great interest and in sustained work on artificial immunity, with a corresponding neglect of studies on the naturally occurring factors of resistance. Workers have accordingly been reluctant to enter this field because of its complexity and because of the lack of adequate methods or tools for the identification and measurement of the factors involved.

Work was concentrated on blood after it was found that it contains substances, both natural and acquired, that are able to destroy bacteria and foreign cells. The easy availability of blood and its relative simplicity when compared to other tissues made it the choice for study by most workers. A wealth of basic studies on the nature and interactions of serum antibodies has resulted that has contributed immensely to our knowledge of acquired immunity. On the other hand, while it has long been known that normal blood serum destroys bacteria and certain other cells and inactivates viruses *in the absence of demonstrable antibody*, the mechanisms and factors involved in these reactions have been ignored. Complement, alone, has received considerable attention mainly because convenient methods are available for its measurement.

Recently we showed that normal human and other mammalian sera contain a protein, properdin, that is an important constituent of the properdin system, a natural defense mechanism of blood.[1, 2] Properdin, in conjunction with complement and Mg^{++}, participates in the destruction of certain bacteria,[1, 3, 4] protozoa,[5, 6] and abnormal red cells,[1, 7-9] and in the inactivation of certain viruses (FIGURE 1).[1, 10, 11] These diverse activities of the properdin system and its presence in normal serum suggest that the properdin system is one of the factors responsible for natural resistance. Indeed, there appears to be a relationship between serum properdin levels and resistance or susceptibility of experimental animals to infection,[2, 12-14] shock,[15-18] and irradiation.[19, 20] Since both properdin and complement can be quantitatively measured, it should be

* This investigation was conducted under the auspices of the Commission on Immunization, Armed Forces Epidemiological Board, and was supported in part by the Office of the Surgeon General, Department of the Army, Washington, D. C.; and in part by a grant from the Lederle Laboratories Division, American Cyanamid Company, Pearl River, N. Y.

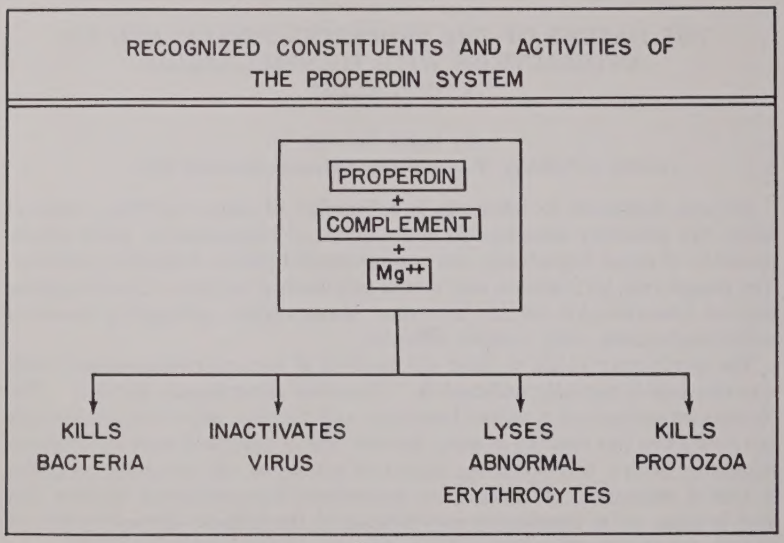

FIGURE 1.

possible to determine the importance of the properdin system in natural resistance.

The present paper, necessarily brief, summarizes existing knowledge on the nature of the human properdin system and some of its interactions and will serve to introduce the more detailed reports on this system that are included in this monograph.

The Nature of the Human Properdin System

Properdin. Human properdin has been purified from zymosan-properdin complexes by elution with buffers of high ionic strength, followed by precipitation, solution, and differential centrifugation.[1, 21] Properdin is a euglobulin with an isoelectric point between pH 5.5 and 5.8, and it contains lipid, carbohydrate, and phosphorus. It is estimated that human properdin comprises not more than 0.02 per cent of the normal human serum proteins. A unit of properdin[21] represents not more than 0.5 μg. of protein nitrogen (TABLE 1).

Purified properdin has a sedimentation constant between 24 to 30 S. It is therefore a large molecule with a molecular weight of over 1,000,000. Upon standing at 1° C. the properdin molecule dissociates, and a family of molecules appears with sedimentation constants of 18, 12, 9, 6, and 3 S. Properdin activity is lost as soon as dissociation to units of 3 S occurs. Thus a properdin preparation originally containing 1,000 units per ml. had dissociated entirely to units of 3 S after standing for 2 months at 1° C. and was inactive both in the zymosan and bactericidal assay. Along these lines it should be mentioned that properdin is inactivated by traces of detergents, soaps, and at pH less than 5.2.

TABLE 1
PROPERTIES OF PURIFIED HUMAN PROPERDIN

Nature	Euglobulin (isoelectric point pH 5.5–5.8)
Lipid	+
Carbohydrate	+
Phosphorus	+
Per cent of serum proteins	0.02–0.03
Units/mg. nitrogen	2000
Sedimentation constants (S)	24–30 $\xrightarrow{1^\circ\ C}$ 18, 12, 9, 6, 3*

* Inactive.

TABLE 2
COMPARISON BETWEEN PROPERDIN AND ANTIBODY

	Properdin	Antibody
Serological specificity	0	+
Presence in normal serum	+	0 or +
Presence in serum fractions	III—1	II or III
Requirements for complexing	C′1, C′4, Mg^{++}	0
Inactivation by complexes	C′3	C′1, C′2, C′4

Saturation with carbon dioxide is also harmful to properdin. The full importance of these observations must await detailed studies correlating all *in vivo* and *in vitro* activities of the properdin system with the dissociation of purified properdin. Also, the sedimentation constant of properdin as it exists in normal human serum remains to be determined. Purified properdin does not contain components of complement or factors concerned with blood clotting. No protease, lipase, amylase, esterase, or phosphatase activities have been found.

Properdin has been found to differ from antibody in many respects (TABLE 2). Properdin combines with diverse and apparently unrelated substances, while antibodies are relatively specific in this respect. Properdin is present in normal serum and even in serum from "germ-free" rats.*[22] Antibodies generally appear in serum in response to antigenic stimulus. Properdin resides in Fraction III-1 (Cohn), while antibodies are usually present in Fraction II but are also found in Fraction III. Properdin requires C′1, C′4, and Mg^{++} for its combination with various agents. Antibodies have no such requirements for combination with antigen. Properdin-zymosan and certain other properdin-polysaccharide complexes inactivate C′3 specifically, while antigen-antibody complexes inactivate C′1, C′2, and C′4, with no effect on C′3.[1] Thus properdin is distinct from antibody in any acceptable sense.

Bactericidal antibody, however, requires complement for its activity and resembles properdin in this respect. Thus properdin may be a primordial type

* These sera were furnished through the cooperation of James A. Reyniers and Morris Wagner, Lobund Institute, University of Notre Dame, Notre Dame, Ind.

of "antibody." More highly developed and specific substances (antibodies) may have been formed during the process of evolution. Properdin, however, is not an "immune substance" within the usual meaning of this term. It does not appear in response to a specific stimulus, nor does it react only with a specific substance. The amount of properdin in normal serum is too small for its variety of activities to be due to specific individual agents pooled in the preparation of the purified protein. Furthermore, the presence of properdin in the serum of "germ-free" rats also substantiates these views.

Serum free of properdin (RP) contains all of the activities of normal serum except that its C′3 is not inactivated by zymosan, and its bactericidal, virus-inactivating, hemolytic, and toxoplasmocidal activities against properdin-sensitive cells or viruses are absent.[1] The addition of properdin to RP restores all of these activities (TABLE 3).

Serum deficient in properdin (RP) is essential to the study of the properdin system and is, in this respect, as important as properdin itself. The system involves 6 known variables: properdin, 4 components of complement, and magnesium.[4, 21] Five of these variables are contained in RP, and any one of them can limit the system and its activity. Indeed, satisfactory RP can be made from only 20 per cent of sera because of the necessity for the maintenance of adequate levels of all of these factors.[21] This presents problems that must be constantly considered in studies on the properdin system and on its mechanism of action.

Complement (*C′*). Complement is a complex of normal serum constituents that participate in many immune reactions.[23-25] While it had been previously thought that antibody was necessary for its action, studies on the properdin system revealed that C′ may also operate in systems that do not require antibody.

C′ is composed of 4 recognized components that are defined by their participation in immune hemolysis, by methods for their inactivation with respect to this reaction, or by methods for their separation from each other. The components of complement are designated by the symbols C′1, C′2, C′3, and

TABLE 3
SERUM DEFICIENT IN PROPERDIN (RP)

Present	
All components of complement	
All clotting factors	
Isoagglutinins	
Antibodies	
Plasmin	
Amylase	
Lipase	
Esterase	
Phosphatase	
Absent	
Bactericidal	Activities against properdin-sensitive cells or viruses
Virus-neutralizing	
Toxoplasmocidal	
Hemolytic	

TABLE 4
SERUM REAGENTS FOR STUDIES ON THE PROPERDIN SYSTEM

Reagent measures	Name	Factors present				
		C'1	C'2	C'3	C'4	P
C'	Normal serum	+	+	+	+	+
C'1	R1	0	+	+	+	0
C'2	R2	+	0	+	+	+
C'3	R3	+	+	0	+	0
C'4	R4	+	+	+	0	+
Properdin	RP	+	+	+	+	0

C'4, respectively.[24] Human serum fractions employed as reagents for the identification and titration of these components are designated R1, R2, R3, and R4 (TABLE 4).[25] R1, for the titration of C'1, consists of the nondialyzable portion of serum soluble at pH 5.4 and ionic strength 0.02 at 0° C. R2, for the titration of C'2, consists of the nondialyzable portion of serum insoluble under these same conditions. R3, the test reagent for C'3, is prepared by treatment of C' with zymosan. R4, for the titration of C'4, is prepared by the addition of hydrazine to C'. While the cofactors concerned with the properdin system resemble hemolytic C' components in most respects, the possibility exists that they may differ from these components.[21] They may reside in distinct chemical structures, or the components of C' may have dual functions.[26, 27] Work on the purification of these factors should help to clarify this problem. The complexity of complement and the extreme instability of its components during purification, however, make this a laborious and difficult task. Only 1 of the components (C'1) has been prepared in a reasonable state of purity.[28-30] Nevertheless, these are problems that must be solved before the exact nature of the properdin system is elucidated.

Magnesium. The activities of the properdin system cannot be demonstrated when the cations of serum are replaced by sodium.[1] The addition of magnesium in normal serum concentrations (10^{-4} to 10^{-5} M) restores these activities. The magnesium requirement is specific. Calcium is completely ineffective. Cobalt and manganese, however, in concentrations between 10^{-2} and 10^{-3} M may replace magnesium in certain of the activities of the properdin system.[4, 11] Such concentrations, being so much greater than those occurring normally in serum, preclude the possibility of these ions taking part in the natural reactions of serum. As far as can be determined, this is the first indication of an *absolute requirement* of a specific cation in the natural defense systems of blood. This unique requirement suggests many experimental approaches to the elucidation of the role of magnesium in the reactions of the properdin system.

Interaction of the Properdin System with Polysaccharides

Human properdin was originally recognized by its ability to combine with zymosan, an insoluble carbohydrate derived from yeast cell walls, to form a

complex that inactivated C'3.[1] This reaction has now been found to occur in 2 distinct stages (FIGURE 2). The first stage involves the stoichiometric combination of properdin with zymosan. C'1 and C'4 (or substances resembling these components of complement) are required along with Mg^{++} for this combination. This reaction proceeds slowly, if at all, at 1° C., but occurs rapidly at 15° C. In the second stage the resultant properdin-zymosan complex (PZ) inactivates C'3 at temperatures above 20° C. and appears to be catalytic. Mg^{++} is also required for this inactivation. Although the evidence is not conclusive, C'1 or a serum factor resembling it appears also to be necessary for the inactivation of C'3 by PZ. Thus the interactions of the properdin system are indeed complex, and their significance must await further studies along these lines.

It is of special interest that, while all 4 components of complement, as well as Mg^{++} and properdin are required for bactericidal, hemolytic, and virus-neutralizing activities against properdin-sensitive cells or viruses, only 2 of the components of complement, C'1 and C'4, and Mg^{++} are required for the complexing of properdin with the cell walls of bacteria. The products of bacteria lysed or killed by the properdin system may remove properdin from serum and inactivate C'3 during this removal. On the other hand, no depletion has been observed either in properdin or complement components during bac-

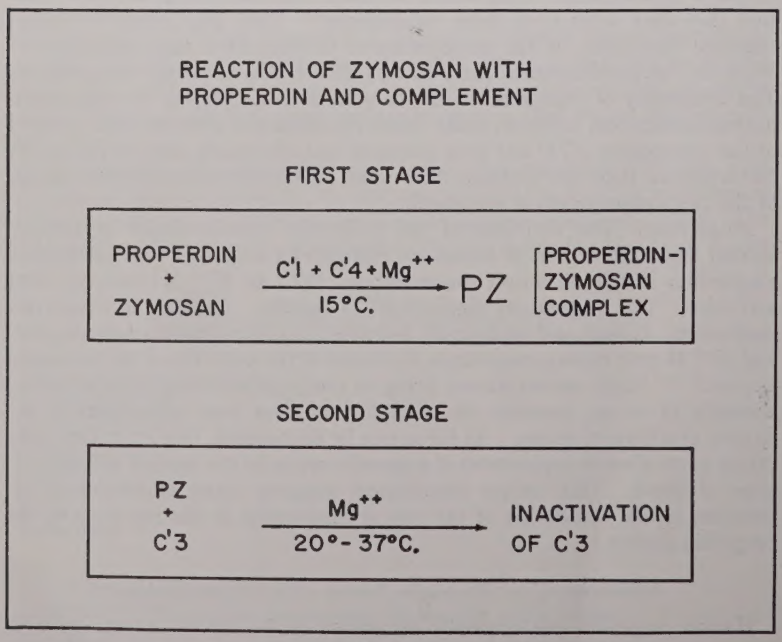

FIGURE 2.

TABLE 5
SUBSTANCES THAT INTERACT WITH THE PROPERDIN SYSTEM

Nature
Polysaccharides or polysaccharide complexes
High molecular weight
Highly branched
Examples and source
Zymosan—yeast
Endotoxins—bacteria
Dextrans—bacterial filtrates
Mucins—animal tissues

tericidal action. It is also noteworthy that a properdin-zymosan complex (PZ) can be formed from mouse and cow sera, even though mouse serum is deficient in hemolytic C′2 and C′3 and cow serum is deficient in C′2. While it is premature to speculate on the significance of these phenomena in host-parasite relationships, it is evident that bacterial products can inactivate or remove substances in serum necessary for bactericidal action of serum.

It has recently been shown that a variety of polysaccharides or polysaccharide complexes of microbial and mammalian origin combine with properdin and inactivate C′3 *in vitro* (TABLE 5) and alter properdin levels *in vivo*.[31] These polysaccharides include bacterial cell walls, endotoxins and lipopolysaccharides, certain dextrans and levans from bacterial filtrates, and lipopolysaccharides, polysaccharides, and mucins from mammalian tissues.*[32] The substances are highly branched and have high molecular weights (10^{-7} and greater). There is no correlation between the toxicity, pyrogenicity, and antigenicity of these substances and their ability to interact with the properdin system. No definite correlation exists between the activities of these materials and any simple physical or chemical property. Polysaccharides with identical repeat units and very similar structures present widely different activities. The active materials contain both α- and β-linkages, furanosidic and pyranosidic units, and interhexose linkages of 1,4; 1,6; 1,3; 2,1; and 2,6 types and combinations of these within the same compound. There also seems to be no dependence on the presence or absence of lipid or protein. Perhaps specific configurations or spatial arrangements of sugar residues may determine the ability of macromolecules, as well as certain bacteria, viruses, and red cells, to interact with the properdin system. In any event, the requirements of C′ and Mg^{++} for the combination of properdin clearly indicate that the mechanisms involved are highly complex and that further comment at this time would be highly speculative.

Experiments have been undertaken to determine whether any physical or chemical change of these polysaccharides occurred following exposure to

* With the exception of certain dextrans and levans, these products are not homogeneous nor composed exclusively of polysaccharides. For the most part these substances, as customarily prepared, also contain nitrogenous, lipid, and other associated materials in varying amounts.
The concept of "purity" for such materials may be misleading. The substances that are the most highly "purified" are probably those that have been most "simplified" in the course of preparation. During isolation and purification they may have been separated not only from accompanying materials but, in all probability, may have been separated also from other moieties with which they are combined *in situ*.

the properdin system.[33] Measurement of such changes would be not only of intrinsic interest but would provide also an approach to the development of a new assay for properdin. No detectable change, however, has thus far been observed in the physical properties of dextrans, levans, or mucins after incubation with fresh human serum at 37° C. In addition, experiments with levans have failed to show a release of fructose from the levan molecule after the above treatment. It is recognized, however, that the polysaccharides tested were, for the most part, heterogeneous, and that the substances that interact with properdin may have been present in amounts too small to permit the demonstration of any change by the analytical procedures employed. Accordingly, attempts are being made to isolate and characterize such products from bacteria, yeast, and mammalian tissues. Exact knowledge of the chemical and physical properties of substances complexing with properdin may give additional information on the mode of action of the properdin system. Such agents may be of value also in the development of new assays for properdin. Furthermore, the alterations in properdin levels that occur *in vivo* following the administration of polysaccharide complexes make it desirable to work with defined substances.

While similar amounts of these agents are required to remove properdin from serum *in vitro* (TABLE 6), lipopolysaccharides derived from bacteria are the most potent agents yet tested for elevating properdin titers *in vivo*.[13] As little as 1 to 10 μg. of these agents causes a marked elevation in properdin titers. This rise appears to accompany increased resistance to infection.[13, 14] On the basis of studies (TABLE 6) on the effect of different polysaccharides of microbial and mammalian origin on the serum-properdin levels *in vivo*,[13, 31] it is now considered that serum-properdin levels may be the result of stimulation or depletion of properdin by both bacterial and host products. Thus an elevation in titer may be due to liberation of small amounts of bacterial or tissue products, while a fall in properdin titer may accompany liberation of large amounts of these substances (FIGURE 3). The identification and characterization of these bacterial and host products should help to clarify and to extend present knowledge on the mechanics of the properdin system and its exact role in natural resistance.

TABLE 6

ACTIVITIES OF CERTAIN POLYSACCHARIDE COMPLEXES ON PROPERDIN

Product	Amount required for	
	removal or inactivation of 8 units of properdin *in vitro*	elevation of mouse properdin levels *in vivo*
	mg.	*mg.*
Bacterial cell walls	0.5–5.0	0.1 –1.0
Bacterial lipopolysaccharides	0.5–5.0	0.001–0.01
Mammalian polysaccharides	1.0–5.0	0.02 –0.1
Zymosan	0.5–5.0	0.1 –1.0

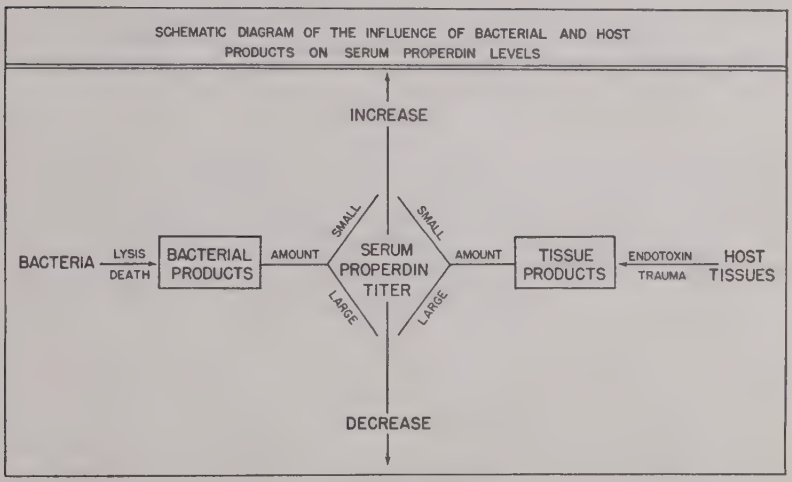

FIGURE 3.

Summary

Properdin, complement, and magnesium ions constitute the recognized components of the properdin system. This system, present in normal serum of man and other mammals, has the ability *in vitro* to destroy bacteria, inactivate viruses, kill protozoa, and lyse abnormal erythrocytes. There appears to be a relationship between serum-properdin levels and the resistance or susceptibility of experimental animals to infection, irradiation, and shock.

Properdin, in a high state of purity, has been prepared by its interaction with zymosan, followed by elution, precipitation, solution, and differential centrifugation. Properdin comprises not more than 0.02 per cent of the normal human serum proteins. It is a euglobulin with an isoelectric point between pH 5.5 and 5.8, and it contains phosphorus, carbohydrate, and lipid. The major portion of freshly prepared properdin solutions has a sedimentation constant between 24 and 30 S. Upon standing at 1° C., a family of molecules appears with sedimentation constants of 18, 12, 9, 6, and 3 S. Properdin activity remains constant under these conditions until the proteins dissociate to units of 3 S.

Human properdin was originally recognized by its ability to combine with zymosan, an insoluble carbohydrate complex derived from the cell walls of yeast, to form a complex that inactivated C′3. Two distinct stages have been demonstrated in this reaction: (1) the combination of properdin with zymosan, and (2) the inactivation of C′3 by the properdin-zymosan complex. C′2 and C′3 are not required for the combination of properdin with zymosan or bacterial cell walls, while all 4 components of complement are necessary for the bactericidal, virus-neutralizing, and hemolytic activities of the human properdin system.

Many high-molecular-weight polysaccharides or polysaccharide complexes of microbial and mammalian origin form complexes with properdin *in vitro* and alter properdin levels *in vivo*. The nature of these substances and their interactions with the properdin system are discussed.

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THE DEMONSTRATION OF THE BACTERICIDAL ACTIVITY OF THE PROPERDIN SYSTEM*

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The properdin system consists of properdin, all 4 components of complement and magnesium.[1] The purpose of the first section of this paper is to describe briefly the experiments that demonstrate that the properdin system has bactericidal activity. Since these experiments are published in full elsewhere,[2] complete descriptions of methods, materials, and results are omitted.

For the majority of the experiments presented, a strain of *Shigella dysenteriae* was used as the test organism because antibody to it is rarely found in normal human serum in the Cleveland, Ohio, area. Bactericidal activity of the materials tested was determined by incubating serum or reagent inoculated with a small volume of organisms suspended in buffer. Following incubation, bacterial survival was estimated by colony counts.

The requirement for properdin in the lysis of bacteria could be demonstrated simply by the incubation of a bacterial inoculum in serum and serum reagents. Fresh serum by itself was bactericidal. RP, serum deficient in properdin, was essentially nonbactericidal. RP with properdin added in its normal concentration in serum (5 units per ml.) had bactericidal activity similar to the original serum. Properdin by itself was not bactericidal, and has been shown to be without bactericidal effect, even in concentrations 100 times its concentration in serum.

The activity of serum and RP with added properdin was a bactericidal phenomenon since, both macroscopically and microscopically, bacteriolysis occurred.

In a similar manner it was shown that the lysis of bacteria by the system also required all 4 components of complement. While serum alone was bactericidal, R1, the reagent lacking C′1, and R2, serum lacking C′2, were nonbactericidal with or without properdin added. The addition of R1 to R2 restored all components to the system and restored bactericidal effect. Similarly the addition of purified C′1 to R1 restored bactericidal activity although C′1 alone was not bactericidal. R3 was not bactericidal, but the addition of partially purified C′3, itself not bactericidal, completed the system, and bactericidal activity was again evident. R4, the serum lacking C′4, was nonbactericidal.

Thereafter it was found that the addition of graded amounts of properdin to RP resulted in graded degrees of bactericidal activity. As little as 0.01 unit of properdin per ml. or 0.2 per cent of the serum concentration produced distinct bacteriolysis. Concentrations between 1 and 10 units of properdin per ml. of RP showed optimal and equal activity. Excessive concentrations of properdin, rather than increasing activity, inhibited the bactericidal process.

* This work was supported by a grant from the Lederle Laboratories Division, American Cyanamid Company, Pearl River, N. Y.

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Since minute concentrations of properdin in RP resulted in distinct bactericidal activity, maximal removal of properdin from serum to be used as an RP reagent in bactericidal and other studies was required. For this reason a double adsorption of serum with zymosan in the preparation of such RP was found necessary. This procedure could be accomplished without inactivation of complement if the amounts of zymosan and the conditions of treatment were carefully chosen.

Double adsorption is carried out by incubating serum with zymosan at 17° C. for 75 minutes. The zymosan is then removed. Fresh zymosan is added to the serum and the mixture reincubated at 37° C. for 30 minutes. Such treatment may result in a decrease in the C'3 titer of the serum. If the C'3 titer of the original serum is high, however, the decrease does not necessarily result in a significant loss of bactericidal activity in the RP when properdin is again added. Generally 10 to 20 per cent of random sera make satisfactory RP for bactericidal studies if great care is taken in preparation.

As with other properties of the properdin system, the bactericidal effect is temperature-dependent. It was found that bactericidal activity did not occur at incubation temperatures below 15° C., partial bacteriolysis appeared between 17° and 20° C., and the activity was complete at temperatures above 20° C.

The bactericidal activity also requires magnesium. The removal of magnesium and calcium from the system resulted in complete depletion of bactericidal activity. The addition of graded amounts of magnesium restored activity in a graded manner, and maximal activity reappeared at magnesium concentrations similar to those occurring naturally in serum. Calcium neither restored activity nor enhanced the effect seen with magnesium alone.

Some 44 strains of a variety of genera of bacteria were tested to determine their susceptibility to the bactericidal activity of the properdin system. These included strains of *Shigella*, *Salmonella*, *Escherichia coli*, *Aerobacter aerogenes*, *Proteus*, *Pseudomonas*, *Paracolobactrum*, and *Bacillus subtilis*. The strains could be divided into 3 general groups. Twenty strains were killed in fresh serum and by RP with properdin added but were viable in RP alone and were classified as susceptible. Fourteen strains were viable in serum and RP with added properdin and were therefore classified as resistant. Ten strains were killed in RP alone, as well as in serum and RP with added properdin, but were viable in properdin alone. Since their susceptibility to the system could not be determined, they were classified as unknown.

The division of strains into susceptible and resistant groups should not be viewed uncritically. Such a division is necessarily arbitrary, and gradations between such groups exist. Indeed, the sensitivity of the strains may depend, in part, upon the type of culture used in the preparation of the bacterial suspension. With some strains, 5-hour broth cultures were consistently more sensitive than 18- to 24-hour slant cultures. It was found, however, that sensitivity to the properdin system *in vitro* was a consistent characteristic of the strain. This sensitivity was a property of the strain, however, not of the species, since most genera contained both resistant and sensitive strains.

Summary and Conclusions

These experiments demonstrate findings that indicate that the properdin system has bactericidal activity. All components of the properdin system (properdin, all 4 components of complement and magnesium) are required for this activity. If any factor is missing, bactericidal activity is lost and, if the missing factor is replaced, bactericidal activity is restored. These and other characteristics of the properdin system distinguish it from systems requiring antibody.

The widespread occurrence of the properdin system in mammalian serum and the variety of bacteria affected by it suggest that the properdin system may be one of the many factors involved in natural resistance.

Acknowledgments

The authors are indebted to Carol Schoenewald and Livia Blum for their valuable technical assistance in these studies.

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THE KINETICS OF THE BACTERICIDAL ACTIVITY OF THE PROPERDIN SYSTEM

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During the course of the experiments demonstrating the bactericidal activity of the properdin system[1] it appeared that while bacteriolysis by the system required properdin and complement, these factors were not necessarily "used up" or inactivated by the bacteriolytic process. It was also noted that, at certain properdin concentrations, small changes in the amount of properdin in RP affected the rate of bacteriolysis markedly. These observations have been extended, and work is at present in progress to determine the parameters of the kinetics of the bactericidal activity of the properdin system. The purpose of this second section of this paper is to present briefly some preliminary observations on the kinetics of the bactericidal process that are to be published *in extenso* elsewhere.[2]

In all experiments, an 18-hour slant culture of *Shigella dysenteriae* was used as the source of the bacterial suspension for the test procedure. Human sera and serum reagents were used throughout. Bactericidal activity was determined from colony counts.

It can be shown that while the act of bacteriolysis requires all 4 components of complement, the bacteriolytic process does not appear to produce any demonstrable change in the concentration of these components in serum. Even with large inocula of bacteria, while there may be a slight decrease in the amount of properdin available for the standard assay, the decrease is not sufficient to decrease the bactericidal activity, and may be related to interference in the assay by the products of bacteriolysis rather than to actual properdin depletion itself.

This can be demonstrated for the bactericidal process by the repeated bacterial inoculation of the same serum. The rate of bacteriolysis following each inoculation appeared to be identical in each of 3 consecutive inoculations. Thus the bactericidal activity of the serum did not appear to be diminished by the prior occurrence of the bacteriolytic processes.

The findings that the components of the properdin system were not consumed in the bactericidal process and that the rates of lysis with repeated inoculation were similar suggest that the factors necessary for lysis existed in such excess that a limiting process was not attained, or that the rate of lysis at any one time was an inherent property of the bacterial population of the inoculum. To test this hypothesis, careful studies have been made relating bactericidal activity to properdin concentration. The relationship between properdin concentration and the per cent of the bacterial population killed is not a linear or first-order reaction. Transformation of the properdin concentration to a logarithmic function produces a characteristic S-shaped curve. If, how-

ever, the susceptibility of the bacterial population is considered to be a normally-distributed characteristic, and the per cent of the bacterial population killed is converted thereafter to a normally distributed function by probit transformation, a linear relationship can be demonstrated.

The closeness of fit of the logarithm of properdin concentration to the probit of the percentage of the bacterial population killed resembles that of many other bactericidal phenomena and is entirely consistent with the hypothesis that the bacterial population employed contains a range of organisms with normally distributed graded susceptibility to the properdin system. The degree of lysis with concentrations up to 0.3 units of properdin per ml. of RP appears related closely to such population variability.

If the degree of bacteriolysis is dependent upon the relative proportions of susceptible bacteria in the inocula, as suggested by these experiments, then the relative rate of lysis should be independent of the size of the inoculum.

This hypothesis can be evaluated by taking limiting concentrations of properdin in RP and using inocula varying in bacterial concentration by ten thousandfold. The relative rate of lysis under these conditions has been found to be independent of the size of the inoculum. The data obtained in this manner do not resemble linear or first-order reactions. Consideration of the fact that the bacterial population contains normally distributed variance (by probit transformation of the per cent of the bacterial population killed), however, again reduces the relationship to linearity. By this transformation it has been found that there is a direct relationship between the probit per cent killed and time, independent of the size of the inoculum.

The relative rate of bacteriolysis by the properdin system at uniform properdin concentrations appears, therefore, to be independent of the size of the inoculum and dependent upon normally distributed variability within the bacterial population used. The amount of lysis, while dependent in part upon the concentration of properdin, is influenced markedly by similar variance within the population of the bacterial inoculum. The effect of such intrinsic bacterial variability in the determination of the relationship between properdin concentration and the rate of bacteriolysis can be avoided by using a standard inoculum and choosing a uniform per cent of the bacterial population killed as a reference point.

By relating the probit per cent of the bacterial population killed at varying times to the logarithm of the properdin concentration, a family of curves can be obtained. Similarly, by relating the probit per cent of the bacterial population killed to time, a family of curves for various properdin concentrations is apparent.

A combination of such data from multiple determinations in simultaneous experiments allows an estimate of the time at which 50 per cent bacteriolysis occurs with various properdin concentrations. The time needed to produce 50 per cent lysis is essentially an index of rate, independent of the intrinsic variance of the bacteria.

From such calculations it has been found that at properdin concentrations greater than 0.3 units per ml. the time required to produce 50 per cent bacteriolysis changes little with increasing properdin concentration. The rate

appears fairly constant. Other factors, probably including the complement content of the serum, appear to be limiting. At properdin concentrations of less than 0.3 units per ml. of RP, however, there is a marked change in rate with varying properdin concentration, and a linear relationship between the logarithm of properdin concentration and the time required to produce 50 per cent bacteriolysis becomes evident. This linear relationship between the logarithm of properdin concentration and time for 50 per cent lysis to occur when the interfering factor of bacterial variance is eliminated suggests a relatively simple process.

Summary and Conclusions

The kinetics of the bactericidal process indicate that the degree and rate of bacteriolysis is in part dependent upon a normally distributed variability in susceptibility to the properdin system of the bacterial population of the strain used. In this respect the quantal response of bacteriolysis by the properdin system resembles many other bactericidal processes.

It has been found that sensitivity or resistance to the properdin system *in vitro* is a property of the strain, not of the species:[1] the kinetics of the bactericidal process suggest that, within limits, a single strain also contains both relatively resistant and relatively sensitive bacteria.[2] Thus the susceptibility of bacteria to the properdin system appears to be an intrinsic property of the strain, not of the species, and of the bacterium within the population of the strain, not of the strain itself. Work is at present in progress to determine factors involved in this attribute of the bacterium.

While the quantitative data relating to properdin concentration in these *in vitro* studies may have little relationship to the properties of the properdin system *in vivo*, the qualitative aspects relating to the bacterium may well apply to animal experiments.

It appears possible, for example, that if the bacterial flora in the animal population to be tested are predominantly properdin resistant, stress that allows such flora to invade the animal might not yield a satisfactory model for the determination of the influence of the properdin system in protection against such stress. The property of susceptibility to the properdin system relates to the strain, not to the species, since strains within a species may be either resistant or sensitive to the system. This fact suggests that in challenge experiments a properdin-sensitive strain of bacteria for the challenge may be advantageous for the demonstration of the effect of the properdin system on such a challenge. Even if the susceptibility of the organism to the properdin system is shown, however, in interpreting data it may be worth while considering that the property of susceptibility to the properdin system of a strain of bacteria is not necessarily an all-or-none phenomenon and may be related to the individual bacterium in the strain, not to the strain itself.

Acknowledgments

The authors express their indebtedness to Carol Schoenewald, Gloria Schwarz, and Livia Blum for their valuable technical assistance, and to Sadja Silberger,

a Western Reserve University medical student, for allowing the inclusion of some of her data.

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INACTIVATION OF VIRUS BY THE PROPERDIN SYSTEM*

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Potentiation of viral neutralization by addition of unheated normal serum to specific immune serum or by the use of unheated serum containing antibodies has been described for several viruses.[1-8] In addition, the inactivation of vaccinia virus by normal rabbit serum[9] and influenza A virus by normal guinea pig serum[10] has been reported. From these studies it has been concluded that complement alone or in conjunction with specific antibody neutralizes certain viruses.[1-10]

Investigations reported in 1949[11] showed that normal fresh human, guinea pig, rabbit, and mouse sera contained a heat-labile component that combined with and neutralized influenza A, influenza B, mumps, and Newcastle disease viruses. The reaction was temperature-dependent and was considered to require calcium. The data indicated that, whereas hemolytic complement was necessary for inhibition of virus, there was another heat-labile factor present in serum that combined with virus and was essential for viral inactivation. The evidence suggested that the heat-labile factor was a protein, but further purification and identification was not accomplished.[11] The work of others was in agreement with these findings.[12-14]

Isolation of the normal serum protein component, properdin, and description of the properdin system by Pillemer and his co-workers[15] have suggested that the components of the heat-labile inhibitor of viral activity might be related to or identical with the factors that comprise the properdin system. This relationship was found to exist.[15, 16] It is the purpose of this paper to summarize the experiments that equate the heat-labile inhibitor of fresh normal serum to the properdin system. These studies are described in detail elsewhere.[16]

Results

The properdin system was described from studies carried out with human serum.[15] Human serum was therefore used to determine whether the properdin system inactivated virus and whether this serum system was identical with the heat-labile serum inhibitor. For these studies it was considered essential to employ a virus that had the following characteristics: (1) it could be readily and accurately quantitated; (2) its specific antibodies were not usually present in human sera; and (3) heat-stable inhibitors of its activity were either not detectable or were present in low concentration in human sera. Newcastle disease virus, an agent propagated conveniently in chick embryos and measured accurately by its capacity to hemagglutinate chicken red blood cells, fulfilled

* This investigation was conducted under the sponsorship of the Commission on Acute Respiratory Diseases, Armed Forces Epidemiological Board, and was supported in part by the Office of The Surgeon General, Department of the Army, Washington, D. C.; and, in part, by grant No. H-1643-C from the National Heart Institute, National Institutes of Health, Public Health Service, Department of Health, Education, and Welfare, Bethesda, Md., and Lederle Laboratories Division, American Cyanamid Company, Pearl River, N. Y.

these criteria. The materials used and details of the techniques employed in this investigation are described elsewhere.[16]

TABLE 1 presents in detail an experiment that demonstrates the inhibition of hemagglutination of Newcastle disease virus, hereafter termed NDV, by fresh but not heated human serum. To avoid small quantities of heat-stable inhibitor, the reagent to be tested was mixed with an equal volume of virus. The mixture was incubated at 37° C. for 30 minutes, following which, serial twofold dilutions of the mixture were prepared and chicken red blood cells were added to each tube. The hemagglutination titer was determined after 1 hour at 4° C. This experimental procedure took advantage of the fact that virus once combined with the heat-labile inhibitor does not dissociate when diluted,[11] whereas the heat-stable viral inhibitor forms a reversible inhibitor-virus complex. The titer was the same whether NDV was mixed with saline or with serum heated at 56° C. for 30 minutes. Mixture of virus with fresh unheated serum, however, resulted in a sixteenfold reduction of the hemagglutination titer, expressed as the "inhibition index." This reduction was equivalent to inactivation of over 93 per cent of the virus. By definition, the "inhibition index" (titer of control/titer with test reagent), when no inhibition of virus is measured, is 1. Since an index of 1 indicates no inhibition of virus, it is reported hereafter as zero.

Properdin requirement for inhibition of virus. To determine whether properdin was essential for inhibition of NDV by the heat-labile inhibitor in fresh serum, sera deficient in this single component were prepared by adsorption with zymosan.[15] The effect of such adsorption on the inhibition of virus is shown in TABLE 2. A single adsorption at 15° C. for 30 minutes using 3 mg. of zymosan per ml. of serum lowered the properdin content to 2 units per ml. of serum. This decrease in properdin concentration, however, did not reduce the amount of virus inhibited. A second adsorption under the same conditions lowered the properdin titer below a measurable level and induced a considerable decrease in viral inhibition. A third adsorption at 37° C. for 30 minutes removed all detectable inhibitor. Serum rendered deficient in properdin has been termed RP.[15]

TABLE 1

EFFECT OF FRESH HUMAN SERUM ON HEMAGGLUTINATION TITER OF NEWCASTLE DISEASE VIRUS (NDV)

Reaction mixture*		Hemagglutination titration							Virus inhibited	
Virus	Reagent	8†	16	32	64	128	256	512	Inhibition index‡	%
NDV	Saline	+	+	+	+	+	§	0	—	—
NDV	Fresh serum	+	⊞	0	0	0	0	0	16	93.75
NDV	Heated serum**	+	+	+	+	+	⊞	0	0(1)	0

* Incubated at 37° C. for 30 minutes.
† Reciprocal of final dilution of NDV.
‡ Titer of control/titer-test reagent.
§ End point.
** Fifty-six degrees C. for 30 minutes.

TABLE 2
CONDITIONS FOR PREPARATION OF RP

Times serum adsorbed with zymosan*	Temperature, °C.†	Properdin units	Hemagglutination titer of reagent NDV mixture	Inhibition index
0		8	1:8	64
1st	15	2	1:8	64
2nd	15	<1	1:128	2
3rd	37	<<1	1:256	0(1)

* Three mg. zymosan/ml. serum.
† Thirty minutes adsorption period.

TABLE 3
PROPERDIN REQUIREMENT FOR INHIBITION OF NEWCASTLE DISEASE VIRUS (NDV) BY FRESH SERUM

Serum reagent tested	Hemagglutination titration		
	Titer	Virus inhibited	
		Inhibition index	Per cent
Heated* serum	1:128	0	0
Fresh serum	1:16	8	87.5
RP	1:128	0	0
RP + P	1:32	4	75.0
P	1:128	0	0

* Fifty-six degrees C. for 30 minutes.

To establish that properdin was essential in inhibiting viral activity it was necessary to determine whether antiviral action could be restored by the addition of properdin to a serum from which properdin had been removed (RP). The results of one experiment are summarized in TABLE 3. The capacity to inhibit hemagglutination was restored to the serum, although full antiviral function was not reinstated by addition of the same amount of properdin that was present in the original fresh serum. This was frequently the case, and it was probably the result of a reduction in C'3 titer by multiple zymosan adsorptions.[17] The converse also occurred, as demonstrated in TABLE 4. In this experiment viral inhibition was measured by infectivity titrations in the allantoic sac of the chick embryo. These results indicate the capacity of the heat-labile inhibitor to reduce infectivity of NDV. These data are presented to point out that, whereas adsorption of properdin from serum reduced its viral-inhibitor content, many of the RP's employed still retained some inhibitory action even though properdin could not be detected by the hemolytic assay method.[18] In these RP preparations there was no reduction in C'3 titer. Complete inhibitory function, comparable to that of the fresh serum, was attained when properdin was added to the RP. These data clearly implicate properdin as a serum component essential for inhibition of NDV by fresh serum. They also point out that a full ration of C'3 is essential to obtain maximum viral inhibition, even with the addition of a large quantity of properdin.

TABLE 4

PROPERDIN REQUIREMENT FOR INHIBITION OF INFECTIVITY OF NEWCASTLE DISEASE VIRUS (NDV) BY FRESH SERUM

Reagent tested*	Infectivity titer, log	Inhibition index, log
	E.I.D.$_{50}$†	
Buffer	−9.5	
Fresh serum	−5.3	4.2
Heated serum‡	−9.2	0.3
RP	−8.5	1.0
RP + properdin	−5.0	4.5
Properdin	−9.3	0.2

* Reagent + NDV incubated at 37° C. for 30 minutes.
† E.I.D.$_{50}$ = 50 per cent embryo-infectious doses.
‡ Fifty-six degrees C. for 30 minutes.

TABLE 5

COMPLEMENT (C′) REQUIREMENTS FOR INHIBITION OF NEWCASTLE DISEASE VIRUS (NDV)

Reagent tested*		Virus inhibited	
Component C′ absent	Properdin units	Hemagglutination inhibition index	Infectivity inhibition index log
None	8	16	3.0
C′1(R1)	0	0	0.0
C′1(R1)	7	0	0.1
C′2(R2)	2–4	0	0.5
C′3(R3)†	0	0	1.2
C′3(R3)†	7	0	1.4
None (C′3(R3) + C′3)	7	—	2.5
C′4(R4)	8	0	0.3
None (R1 + R2)	5	8	—

* Reagent + NDV incubated at 37° C. for 30 minutes.
† Ten per cent of original concentration of C′3 remained.

Requirement for complement components on inhibition of Newcastle disease virus. To determine whether inhibition of NDV by fresh serum required all components of complement as well as properdin, sera deficient in different components of complement were tested for viral inhibitory action. It is clear from the data (TABLE 5) that all components of complement as well as properdin were essential for viral inhibition. Serum made deficient in C′3 (R3), while less inhibitory than whole serum, retained some inhibitor as measured by infectivity titrations, probably because C′3 was not completely depleted. Properdin, although presumably present, could not be detected by the hemolytic assay.[18] Properdin added to a level comparable with that measured in normal serum did not increase viral inhibition. When purified C′3 was added to the R3 the inhibitory activity was restored. Inhibitory activity of serum was also reinstated when reagents from which C′1 (an R1) or C′2 (an R2) had been removed were recombined (the addition of "end-piece" to "mid-piece"). Total restoration did not result because the addition of R1 to R2 resulted in a twofold dilution in respect to whole serum.

Thus, as with other activities of the properdin system,[15, 17, 19] all constituents

of complement in addition to properdin were essential for inhibition of NDV.

The role of magnesium in the inhibition of Newcastle disease virus. In addition to properdin and complement, the properdin system requires magnesium for all of its described activities.[15] To equate inactivation of NDV by the heat-labile serum inhibitor to the properdin system, the cation requirements for viral inhibition were investigated. Serum was mixed with a cation exchange resin, Amberlite IRC-50 in the sodium cycle, to remove magnesium and calcium.[20] As shown in TABLE 6, resin-treated serum deficient in these cations did not inhibit hemagglutination by NDV. Addition of calcium to a concentration of 5 mM. did not restore antiviral activity to the serum. When a similar concentration of magnesium was added, however, full inhibitory activity was restored. When both calcium and magnesium were added, inhibition was not enhanced beyond that obtained with magnesium alone.

The quantitative relationship between magnesium concentration and amount of virus inhibited was investigated. Preliminary experiments[21] indicated that a direct logarithmic function existed between the quantity of virus inactivated and the concentration of magnesium used (between 0.02 and 2.5 mM./liter). These data suggest that to inactivate NDV the interactions of magnesium with the other components of the properdin system occur in multiple proportions.

A number of other divalent cations were also tested to determine whether magnesium was a specific requirement for activity of the properdin system in the inhibition of virus. Of the cations tested, only manganese could substitute for magnesium in the inhibition of hemagglutination by NDV. The concentration of manganese required for this function, however, was considerably in excess of that present in the serum of man or animals, precluding the possibility that it might play a physiologic role in natural resistance to infections.

Optimum temperature for inhibition of Newcastle disease virus by fresh serum. Functions of the properdin system have been shown to require temperatures above 15° C.[15, 17] In addition, the optimum temperature for reaction of properdin with zymosan is above 15° C.[15] The thermal requirements for inactivation of NDV by fresh serum were investigated to determine whether they corresponded with those described for other reactions of this system. The results of these studies, summarized graphically in FIGURE 1, indicate that be-

TABLE 6

CATION REQUIREMENT FOR INHIBITION OF NEWCASTLE DISEASE VIRUS (NDV) BY FRESH SERUM

Reagent tested*	Cation present		Inhibition index
	Calcium†	Magnesium†	
Fresh serum	+	+	16
Resin treated serum‡	0	0	0
Resin treated serum‡	+	0	0
Resin treated serum‡	0	+	16
Resin treated serum‡	+	+	16

* Reagent + resin treated NDV incubated at 37° C. for 30 minutes.
† Five mM.
‡ Serum reacted with cation exchange resin (Amberlite IRC-50, sodium cycle).

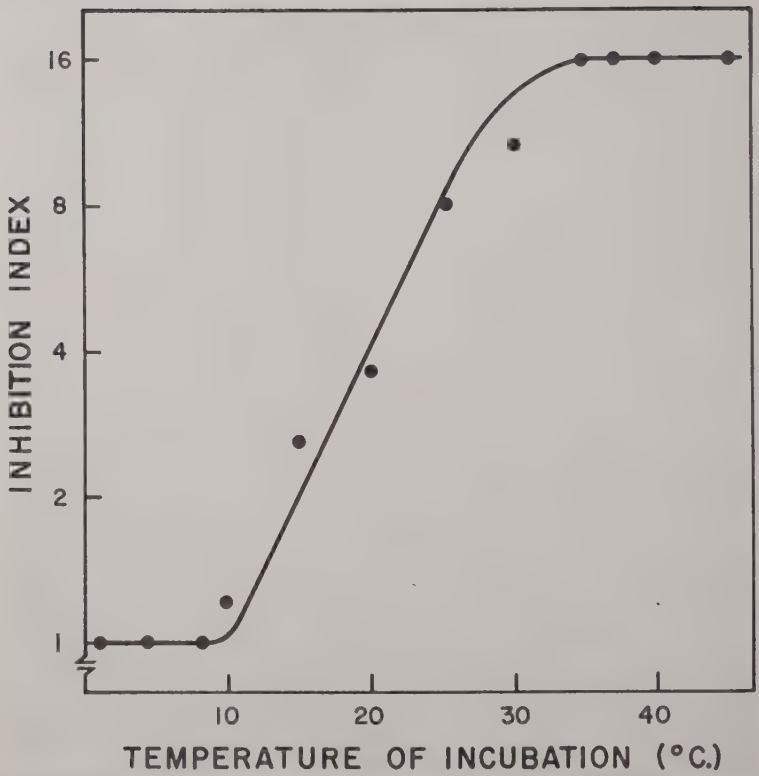

FIGURE 1. Effect of temperature on inactivation of Newcastle disease virus (NDV) by fresh human serum. Serum- and NDV-infected allantoic-fluid mixtures were incubated at the indicated temperatures for 30 minutes. Viral activity was measured by hemagglutination titrations.

tween 10° C. and 35° C. there was a straight line function between the quantity of virus inhibited and the temperature of incubation of the virus-serum mixture.

Quantity of properdin required to inhibit activity of Newcastle disease virus. Previous data suggested that the quantity of properdin in fresh human serum was considerably in excess of that necessary to accomplish inhibition of NDV. Experiments were designed to determine the minimum concentration of properdin required in serum to accomplish viral inactivation. In the first series of experiments properdin was added in increasing amounts to properdin-depleted serum, and the inhibition of viral infectivity was determined. The results are summarized in FIGURE 2. It will be noted that the RP employed was not entirely lacking in antiviral activity. The addition of as little as 0.1 unit of properdin per ml. of serum increased viral inactivation considerably. As increasing quantities of properdin up to 2.5 units per ml. were added, there was a progressive increase in the embryo-infectious doses ($E.I.D._{50}$) of virus

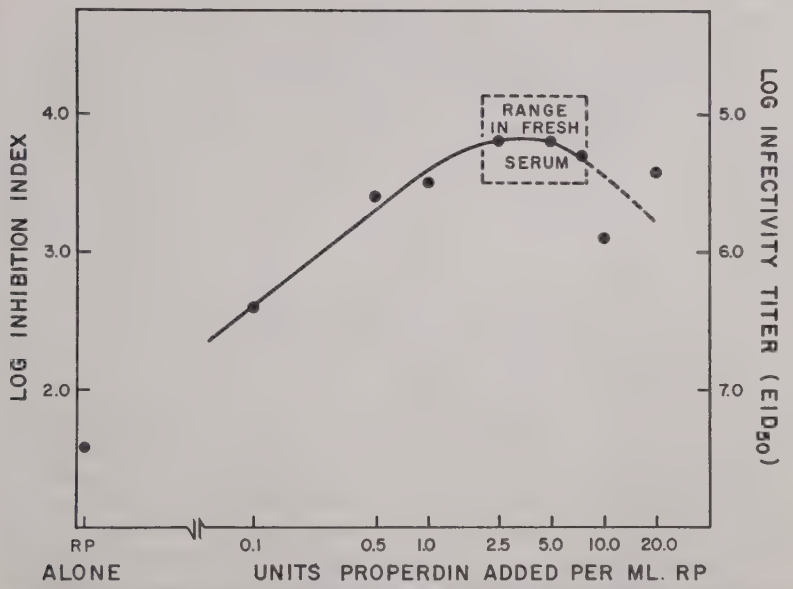

FIGURE 2. The relationship between properdin concentration and the quantity of virus inhibited. Increasing amounts of properdin were added to a serum rendered properdin-deficient by adsorption with zymosan (RP). Final volumes were constant in all mixtures. Infectivity titrations were employed to determine active Newcastle disease virus (NDV).

inhibited. At a concentration of 2.5 units of properdin per ml. of serum the inhibition of virus corresponded to that obtained with the parent serum. Further addition of properdin did not increase viral inactivation. Indeed, the data suggest that excessive amounts of properdin added to serum actually decreased the viral inhibitory activity.

When similar experiments were done, and the inhibition of virus was measured by hemagglutination titrations, similar results were obtained. These studies are summarized in TABLE 7. In this instance the RP employed again had inhibitory activity, although it was considerably less than that obtained with the original serum. Heated RP (56° C. for 30 minutes) and properdin alone did not inhibit hemagglutination by NDV. It is striking in these data that it was necessary to add only exceedingly small amounts of properdin, 0.03 units per ml., to inhibit a quantity of NDV that corresponded to that amount inhibited by the original serum. With concentrations of properdin greater than 0.03 units per ml. there was no increased inactivation of virus.

From the experimental results obtained, it is not possible to determine the absolute minimal concentration of properdin essential to inactivate NDV because the RP's employed all had a residuum of antiviral activity. These data do imply, however, that the concentration of properdin necessary was less than that concentration detectable by current assay procedures and was considerably less than that found in the serum of man under a variety of conditions in health or disease.[22]

TABLE 7

QUANTITY OF PROPERDIN REQUIRED TO INHIBIT HEMAGGLUTINATION OF NEWCASTLE DISEASE VIRUS (NDV)

Reagent tested*	Units properdin added	Inhibition index
Serum	0	64
Buffer	8	0
RP	0	8
Heated RP†	0	0
RP	8	64
RP	2	32
RP	0.5	32
RP	0.125	64
RP	0.03	64
RP	0.008	8

* Reagent + NDV incubated at 37° C. for 30 minutes.
† Fifty-six degrees C. for 30 minutes.

Effect of serum-Newcastle disease virus interaction on properdin and complement levels. Since inactivation of NDV by fresh human serum required properdin, all components of complement, and magnesium, it was important to estimate the role of each of these factors in order to derive hypotheses concerning the mechanism of viral inactivation. Experiments were designed to determine whether or not the active factors in the properdin system were bound when reaction with virus occurred. Fresh serum was mixed with undiluted NDV-infected and normal allantoic fluid, and the mixtures were incubated at 37° C. for 30 minutes. Analyses were then made to determine the hemagglutination titer as well as the concentration of properdin, hemolytic complement, and complement components in each mixture. The results of 3 experiments are summarized in TABLE 8. These data indicate that with inactivation of virus the amount of properdin measured by the standard assay procedure decreased without a significant reduction in the titer of complement or its individual components. These findings correspond to those previously reported from an investigation of the heat-labile inhibitor of guinea pig serum, in which the inhibitor was bound by influenza virus without a decrease in titer of complement.[11] It might be suggested from these data that inactivation of NDV is accomplished by the combination of virus with properdin. The reaction requires all components of complement, although they are not combined with the infectious agent in detectable quantities.

The effect of ion exchange resin upon the Newcastle disease virus-properdin complex. To inactivate NDV by the properdin system, magnesium or manganese were required. Previous investigation of the heat-labile inhibitor in fresh guinea pig serum indicated that inactivated virus could be dissociated from inhibitor by chelation of the divalent cation with sodium citrate.[11] The reaction that yielded reactivated virus was not an immediate one, and it was temperature-dependent.[11] Preliminary experiments were carried out to determine whether NDV inhibited by the properdin system could be similarly reactivated by removal of cation from the reaction mixture.[21] To a serum-NDV mixture in which 93.5 per cent of the virus was inhibited, an equal volume of the ion

Table 8

Effect on Serum Properdin and Complement Titers of Incubation with Newcastle Disease Virus (NDV)

Reaction mixture*	Inhibition index	Titer components of properdin system					
		P	C′	C′1	C′2	C′3	C′4
Serum + NDV	64	4	80	400	270	270	1070
Serum + allantoic fluid	—	7	90	560	290	290	1010

* Incubated at 37° C. for 30 minutes.

exchange resin, Amberlite IRC-50 in the sodium cycle, was added. The mixture was incubated for varying periods at 37° C. and at room temperature, centrifuged, and the hemagglutination titer was determined. The results of these experiments indicated that there was no immediate increase in hemagglutination titer of the mixture, nor was an increase in free virus detectable after incubation at 37° C. for 30 minutes. When the serum-virus-resin mixture remained at room temperature (22° to 24° C.) for 18 hours and the resin was finally removed by centrifugation, a marked reappearance of hemagglutinating virus was measured. Although a marked and significant quantity of virus (35 to 50 per cent) was dissociated from the inactive combined state, in no experiment was it possible to reactivate all of the inhibited NDV. These data suggest that either the capacity to remove magnesium from the reaction was limited under the conditions of these experiments or the remainder of the virus was completely inactivated. Furthermore, these data suggest that magnesium is an important linkage in the properdin-NDV combination. Finally, the results of these experiments clearly imply that all of the virus inhibited is not irrevocably inactivated by the properdin system as measured by hemagglutination.

Discussion

The data presented indicate that the heat-labile virus-inhibitory system of fresh normal human and animal sera[11] is indeed the properdin system.[15] It had been demonstrated previously that inhibition of influenza, mumps, and Newcastle disease viruses by unheated serum required a heat-labile factor in addition to all components of complement and a divalent cation thought to be calcium.[11] The results of this investigation indicate that the unknown heat-labile factor was probably properdin and that the divalent cation was magnesium. That the heat-labile inhibitor can be equated to the properdin system was further demonstrated by the similarity of experimental results that showed corresponding temperature dependence, adsorption of properdin or heat-labile inhibitor without significant reduction in complement or complement component titers, and the reactivation of virus by reducing the availability of magnesium to the virus-properdin (or inhibitor) complex.

A consideration of the data presented permits one to raise hypotheses concerning the possible mechanism by which the properdin system inactivates NDV and probably other viruses affected by fresh normal serum. The role of

complement is not clear. That all components are essential for viral inactivation, however, appears unquestionable. Indeed, any reduction in C'3 at least rendered a serum incapable of maximum inhibition regardless of the concentration of properdin available. Conversely, a serum with its full ration of complement components attained its maximum effectiveness with an extremely small amount of properdin. Despite this essentiality for complement, the components could not be demonstrated to be bound to virus. The concentration of properdin, on the other hand, was reduced when virus was inactivated.

These data suggest that NDV was inactivated as a result of the combination of virus and properdin in the presence of magnesium and the components of complement. The dissociation of the complex with liberation of active virus probably resulted from preferential binding of magnesium by an ion exchange resin. These results imply that magnesium was not only necessary for association of properdin with NDV, but also that magnesium was essential to maintain this combination. This introduces the notion that virus-properdin association is linked by magnesium. Removal of magnesium breaks the linkage, and active virus is again detectable.

The experimental results imply that NDV, as well as other viruses inactivated by fresh normal sera, is affected by the properdin system by a mechanism similar to its bactericidal, hemolytic, and antitoxoplasmic effects.[17, 19, 23, 24] These studies raise the provocative implication that the viral particles of the species affected may have chemical or physical configurations similar to or identical with certain bacteria, abnormal erythrocytes, protozoa, and complex polysaccharides. Indeed, the data suggest that NDV at least reacts with the properdin system in a manner that might be likened to the reaction between zymosan and properdin. It is clear, however, that all viruses do not react with the properdin system, nor do all bacteria and all red blood cells. Although the list of animal viruses inactivated by fresh normal serum alone (influenza A and B, mumps, Newcastle disease, and vaccinia)[9, 11] or in conjunction with antibody (Rous sarcoma, western equine, herpes simplex, and dengue viruses)[2-4, 6-8] is impressive, many important agents such as poliomyelitis[25] and the newly described respiratory viruses[26] are not inactivated by fresh normal serum.

There is no evidence available yet as to the role that the properdin system might play in either natural or acquired immunity in viral infections. One may hypothesize that infection with influenza, vaccinia, or herpes virus remains localized and that demonstrable viremia is rare because of the inactivation of these agents by the properdin system. This would imply that the occurrence of detectable viremia would result from a reduction in properdin or complement levels. With properdin, at least, this seems unlikely because so little of this serum component is required to effect maximum viral inactivation. Moreover, viremia is an important phase in the pathogenesis of a disease such as mumps. This agent, however, is readily inactivated by the heat-labile inhibitor system in human sera.[11]

Much further study is necessary in order to determine whether this extremely interesting inhibitory system, the properdin system, does play an active role in the protection of man and animals from certain viral infections. From a teleo-

logical viewpoint it would seem odd, however, that this complex, effective inhibitory system should be without any beneficial role.

Summary

The properdin system, comprised of properdin, all components of complement, and magnesium, was demonstrated to be responsible for the capacity of fresh human serum to inactivate Newcastle disease virus (NDV). Relatively small quantities of properdin were required for viral inactivation. A mixture of NDV-infected allantoic fluid and fresh human serum resulted in inactivation of virus and a decrease in properdin titer, but did not effect a significant reduction in titer of any of the components of complement. Inactivation of NDV was partially reversed by the ion exchange resin, Amberlite IRC-50 in the sodium cycle.

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Discussion of the Paper

Doctor L. Levine (*Division of Laboratories and Research, New York State Department of Health, Albany, N. Y.*): All of you who have worked with animal viruses can appreciate the amount of work that is represented by Doctor Ginsberg's report. Realizing the difficulties inherent in studies with animal viruses, Helen Van Vunakis, James Barlow, and I are investigating the neutralization of bacterial viruses by fresh sera. We have used T_2 phage, one of the T-series of bacteriophage active on *Escherichia coli* B. This virus can be obtained in highly purified form in gram quantities, and 1 stock solution of

virus is stable over a period of years. It can be assayed easily with desirable precision. In addition, this virus can be radioactively labeled, using P^{32} for the nucleic-acid portion and S^{35} for the protein coat. In this manner, studies on the mechanism of neutralization are feasible.

We have found that $T_{2r}+$ phage is inactivated by fresh human, pig, cow, rat, rabbit, mouse, and guinea pig sera.[1] Experiments on the role of properdin and complement (C′) in phage neutralization were conducted with human-serum reagents lacking C′1, C′2, C′3, C′4, and properdin. No neutralization was observed with any of these reagents. Recombination of any 2 C′ reagents that restored C′ activity, as judged by hemolysis, also restored the viricidal activity. When fresh serum was heated at 56° C. for 15 minutes, the phage-neutralizing activity was destroyed. Viricidal action was eliminated by the addition of the chelating agent, ethylenediaminetetraäcetate, indicating a requirement for divalent cations and further implicating the C′ system. A reagent lacking properdin (RP) prepared by treating fresh human serum with zymosan exhibited no neutralizing activity. Purified properdin (sent to us by Louis Pillemer), when added to RP, restored viricidal activity, and the degree of neutralization depended on the quantity of purified properdin added.

The effect of pH, ionic strength, temperature, and metals on the neutralization of T_2 phage by fresh human serum was studied. This system appears to be *p*H and ionic-strength sensitive with the optimum *p*H being between 6.7 and 7.1, and the optimum salt concentration 0.126 osmolar. The divalent cations Mg^{++}, Mn^{++}, and Co^{++} enhance the phage-neutralizing activity. The rate of reaction is temperature-dependent, with the optimum between 36° C. and 42° C. Preliminary kinetic experiments show inhibition at high concentrations of sera.

These studies on the neutralization of bacteriophage by fresh human sera suggest that the reaction may be an indicator of properdin levels. The feasibility and reliability of using phage neutralization to assay for properdin is under investigation.

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THE RELATIONSHIP OF *TOXOPLASMA* ANTIBODY ACTIVATOR TO THE SERUM-PROPERDIN SYSTEM*

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It was reported in 1948[1] that a heat-labile serum component, since known as "activator," was required in order for neutralizing antibodies to affect *Toxoplasma gondii*. Human serum was found to be the most suitable source of activator because, in the absence of antibody, such serum had no effect upon the parasite. Normal fresh mouse serum also was found not to affect *Toxoplasma*, but neither would it restore the "activator" effect to serum from which this effect had been removed by heating for 30 minutes at 56° C. Since mouse serum is poor in C′2 and C′3, the role of complement in the system was explored further. C′1 and C′2[1] † derived from human complement were restored, both singly and in combination, to inactivated human serum. This restoration resulted in the reconstitution of complement for a hemolytic reaction, but not in the renewal of *Toxoplasma* activator. This experience led to the conclusion that the hemolytic complement, per se, was not the heat-labile serum component required for the functioning of the *Toxoplasma* antibody system. It was learned also that proportionately more activator was required for the functioning of the *Toxoplasma* antibody system than complement was for the hemolysis of sensitized sheep cells.

Human sera were found to differ from other animal sera in another respect. Fresh, normal rabbit, guinea pig, rat, monkey, sheep, cow, horse, and dog sera, in the absence of antibody, were found to kill most of the parasites in a suspension and to destroy their affinity for alkaline methylene blue, which is used as the indicator in the dye test. Specific antibody is presumed not to be present because, when such sera are inactivated prior to examination in the dye test, a negative reaction is generally encountered. Other inactivated animal sera were found to yield significant antibody titers when titrated in the presence of the standard human activator. These reactions are consistent with the patterns observed following experimental infections.

Although this nonspecific antitoxoplasmic affect has received scant attention, there has been some feeling that it might play a role in natural resistance. It has been noted that young animals are more likely to succumb to inoculation with parasites than are mature animals. The latter frequently have nonfatal infections despite the fact that parasitemia can be detected. A prominent exception to this is the mature rabbit, which usually dies following the intradermal introduction of virulent parasites. One could assume that this factor reduces the inoculum to the point where the host has an opportunity to provide himself with sufficient protection to withstand the later dissemination of the parasites. In the case of the intradermal inoculation, the parasites may be

* The work described in this paper was supported by grants from the National Institutes of Health, Public Health Service, Department of Health, Education, and Welfare, Bethesda, Md.

† Supplied by Louis Pillemer.

protected from the serum systems until they are able to multiply. If this explanation is correct, then one would have to assume that the nonspecific factors are produced or increased to a significant degree with the maturation of the animal. Obviously, much remains to be learned about these mechanisms and in view of our awakened interest in the activator, we recently have turned our attention to nonspecific systems.

The activator system, both by accident and design, has been investigated by us from time to time. In the original[1] paper describing the dye test, it was stated that 8.5 per cent saline inhibits the dye test although it does not interfere with the staining of *Toxoplasma*. This observation has been examined further in subsequent studies and it was learned that the action of the activator is inhibited by the presence of NaCl concentrations of 2 per cent[2] or greater. Although it had been customary to destroy the activator effect by incubating serum at 56° C. for 30 minutes, we have since conducted a detailed study of the effect of temperature on human activator and found that the critical temperature is 48° to 49° C., that is, whereas 10 to 12 hours at 37° are required to destroy 50 per cent of the activity, the same point is reached after 4 to 5 hours at 42° C., 2 hours at 45° C. and only 10 to 12 minutes at 47.5° C. At 50° C., 4 to 5 minutes are sufficient, while at 53° C., 2 to 3 minutes and at 56° C., 1 to 2 minutes are sufficient to destroy 50 per cent of the effect. Only 30 to 45 seconds are required at 60° C. In each instance, the activator was held in ice water until it was transferred to a water bath heated to the temperature under study. We believe that the lag is due in part, if not entirely, to the time required to bring the temperature of the test system[2] from approximately 4° C. to the test temperature.

In other experiments it was learned that dialysis of activator against distilled water in the cold for 24 hours did not destroy its activity.[2]

We prepare activator, generally, by removing 500 ml. of blood from a human donor who has no antibodies. The blood is drawn directly into a flask containing glass beads, where it is defibrinated by agitation as it is being removed. Following the completion of defibrination, the blood is transferred to large centrifuge bottles, the cells quickly sedimented, and the serum is removed, pooled, distributed to smaller vials, and rapidly frozen in a dry ice-alcohol mixture. All of these steps are conducted in the cold. We know that activator will remain unaffected at least for 4 years when stored in dry ice, but there is considerable evidence to suggest that 3 to 4 weeks at −20° C. will result in a significant loss of potency.

Several years ago it occurred to us[2] that a simpler way to prepare activator would be to use 1 of the newer plastic bleeding units that contains a resin column to remove the calcium. This proved to be a simple, effective method for collecting serum but, much to our surprise, the activator processed in this fashion turned out to be very poor. Although it was not completely ineffective, it was too inactive to allow for its regular use. We then set up a series of experiments in which the various metals that the resin had removed were reintroduced, and found that when we replaced the magnesium we got a reasonably good return of activity. This led to the impression that magnesium played

a role in the activator system but, unfortunately, this effect could not be produced with regularity.

To sum up, we knew that *Toxoplasma* antibody required the presence of heat-labile serum components in order to exert an adverse effect upon the parasite. In the case of human serum, no measurable effect could be detected unless antibody was present. Mouse serum had no effect unless human serum was added to the system. The fresh sera of other animals could exert a marked effect on the parasite in the absence of antibody, and this effect could be destroyed by heat. The activator of human serum was destroyed at temperatures of 48° to 49° C. by 2 per cent saline, was nondialyzable against distilled water, and could be stored indefinitely in dry ice. There was a suggestion, at least, that magnesium was important for its action. If complement was important, the evidence suggested that that portion which is necessary for the lysis of sensitized sheep cells was not solely involved in the *Toxoplasma* system.

When the properdin system was reported,[3] it was apparent that activator would have to be restudied from this point of view. That this might occur to more than 1 investigator is obvious and, in fact, Grönroos of the University of Helsinki, Helsinki, Finland, conducted such studies, which have been reported.[4] Grönroos was interested in studying the mechanism of the dye test, and he came to the conclusion that activator serum is made up of properdin plus C′2, C′3, and C′4. Although he does not directly state it, the assumption is that he found that magnesium was also required. He seems to think, however, that

TABLE 1

EFFECTS OF VARIOUS PROPERDIN MIXTURES UPON *TOXOPLASMA* IN THE DYE TEST*

Test mixture			Dye test		
Serum	Properdin added units		Stained,** per cent	Unstained, per cent	Interpretation
RP†	0	—	88	12	Negative
RP	25	(Human)	70	30	Negative
RP	10	(Human)	20	80	Positive
RP	5	(Human)	20	80	Positive
RP	2.5	(Human)	10	90	Positive
RP	1.25	(Human)	20	80	Positive
RP	10	(Bovine)	84	16	Negative
RP	4	(Bovine)	50	50	Positive
RP	0.5	(Bovine)	14	86	Positive
Normal serum‡	0	—	96	4	Negative
Saline	250	(Human)	94	6	Negative
Normal serum‡	250	(Bovine)	N.C.§	N.C.	Negative
Saline	250	(Bovine)	N.C.	N.C.	Negative
Neg. serum cont.	—	—	96	4	Negative
Pos. serum cont.	—	—	6	94	Positive

* RH strain of *Toxoplasma* from mouse peritoneal exudate.[1] Peritoneal exudate (0.1 ml.) added to 0.4 ml. of serum or saline. Properdin added as 0.03 ml. or less. Heparin added in final concentration of 1:10,000. 0.1 ml. of *Toxoplasma*-serum-properdin mixture added to 0.1 ml. of antibody-containing human serum which had been diluted 1:8 with saline or a saline equivalent.

** 100 parasites counted. 50 per cent or more unstained = positive effect.

† Human serum lacking properdin but containing hemolytic complement.

‡ Serum inactivated for 30′ at 56° C.

§ Not counted. Almost all stained.

properdin (possibly with C'2, C'3, and C'4) can affect *Toxoplasma* in the dye test in the same way that properdin and immune serum react together. Grönroos infers that given sufficient properdin, one may encounter a positive dye test in the absence of antibody. He also offers the hypothesis that in congenital toxoplasmosis the expression of fetal disease is dependent upon the presence of an inadequate maternal properdin level prior to the appearance of antitoxoplasmic antibodies.

Our own experiences (summarized in TABLE 1) have led to somewhat different conclusions. It is apparent that the complement and Mg^{++} alone do not help antibody activity. Large quantities of either human or bovine properdin, per se, also do not act upon *Toxoplasma*, nor will properdin activate serum whose complement and properdin have been removed by heat inactivation. As in a bactericidal system, the addition of an excess of properdin to the RP serum results in inhibition of the reaction.[5]

In the light of these experiments it appears that the heat-labile serum component labelled "accessory factor" in the original description of the *Toxoplasma* dye test[1] is similar to, if not identical with, the properdin system[3] as it is now defined: properdin + complement + Mg^{++}. The role of C'1 requires further clarification.

If the "accessory factor" is the properdin system, then, in the present state of our knowledge, *Toxoplasma* represents an almost unique situation, since it provides the only example in which the action of an antibody is dependent upon the presence of the properdin system. This phenomenon is either unique for *Toxoplasma*, or occurs with other protozoa as well, or else antibodies have not been recognized when present in the other places where the properdin system is directly active. A final possibility is that perhaps there is some other substance in the properdin system that is not detectable in the nonantibody situations but may be required for the action of *Toxoplasma* antibody.

An analogous system may be present among the viruses for, while it has been demonstrated that the properdin system inhibits certain viruses[6] in the same way that it is bactericidal for some gram-negative organisms, it has also been pointed out[1] that the neutralizing effect of antisera upon dengue virus depends upon the presence of heat-labile serum components. This possible exception is worthy of further exploration.

Acknowledgment

I obtained the data summarized in TABLE 1 in experiments conducted in the laboratory of Louis Pillemer, whose help and advice I acknowledge with pleasure.

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PROPERDIN LEVELS IN INFECTIOUS AND NONINFECTIOUS DISEASE*

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The possibility that the properdin system might be involved in human disease first became apparent when it was found that the properdin system is required for the hemolysis *in vitro* of the abnormal erythrocytes from patients with the chronic hemolytic anemia known as paroxysmal nocturnal hemoglobinuria (PNH).[1, 2] As yet, this is the only instance in which properdin has been shown to interact with an abnormal human tissue, presumably leading to the destruction of that tissue.

Paroxysmal nocturnal hemoglobinuria is a rare hemolytic anemia, the chief characteristic of which is an acquired abnormality of the erythrocyte such that hemolysis occurs *in vitro* in the patient's own or in any other normal human serum. No antibody has been found to be required for this reaction. Thus the defect is in the erythrocyte, and the hemolytic system is a property of normal human serum. Observations previously reported[2] have shown that a normal human serum will hemolyze PNH erythrocytes, but a serum lacking in properdin or any of the components of complement will fail to produce hemolysis. The addition of purified properdin to a properdin-deficient serum restores hemolytic property. Properdin fails, however, to restore hemolytic activity to a serum that lacks any component of complement. The removal of magnesium renders a serum nonlytic for PNH erythrocytes, but the addition of magnesium ions restores the hemolytic property. Thus properdin, the components of complement, and magnesium, which make up the properdin system, are all required for the hemolysis of PNH erythrocytes *in vitro*. This requirement for properdin in PNH hemolysis appears to be unique among naturally occurring systems, since none of the immune hemolytic systems tested have similar requirements. It should be noted, however, that normal human erythrocytes treated with tannic acid require the properdin system for hemolysis *in vitro* in human serum.[11]

During the past 2 years, 4 patients with PNH have been studied.[3] In each of these individuals an extremely low properdin level has been detected at some time during the course of his disease. One patient, a Negro woman, has had severe hemolytic disease for 10 years and has required approximately 100 transfusions. Repeated properdin determinations have been obtained during the last 2 years. During the periods of crisis or intense hemolytic activity, the serum-properdin level has been low. On some occasions during remission, when there was less hemolytic activity, the serum-properdin level was observed to be within normal limits. The mechanism responsible for this lowered properdin level at the time of marked hemolytic activity is unknown. The possi-

*This investigation was conducted under the auspices of the Commission on Immunization, Armed Forces Epidemiological Boards, and was supported in part by the Office of the Surgeon General, Department of the Army, Washington, D. C., and in part by a grant (H1263 C) from the National Heart Institute, National Institutes of Health, Public Health Service, Department of Health, Education, and Welfare, Bethesda, Md.

bility exists that utilization of properdin occurs during the hemolytic process *in vivo*. Properdin is not utilized during the hemolysis of PNH erythrocytes *in vitro*, however, nor has it been demonstrated that properdin combines with the stroma of PNH erythrocytes.

Properdin combines *in vitro* with a variety of polysaccharide substances, including the cell walls or endotoxin from gram-negative bacteria and polysaccharides from certain pneumococci.[4] In addition, a fall in properdin level *in vivo* has been observed in animals following experimental infection, hemorrhagic shock, and whole-body irradiation, states in which death has been attributed to endotoxemia or bacteremia.[5, 6] These data suggested that changes in serum-properdin level might occur in certain human diseases, particularly infections. Accordingly, serum-properdin levels have been determined on a group of patients from the medical and surgical services of University Hospitals of Cleveland, Ohio, and on serum samples submitted from other institutions. These results are admittedly incomplete, and many diseases are not included. As determined by the zymosan assay,[7] the serum-properdin level in most normal individuals is between 4 and 8 units per ml., and it remains constant over many months. No normal individual has had a properdin level of less than 2 units. Since considerable variation between individuals has been noted, greater significance has been attached to changes in the properdin level during an illness rather than to a single determination. Accordingly, serial determinations were done on all patients. It is to be pointed out that the zymosan assay for properdin as it is usually performed measures a minimum of 1 unit per ml. of serum.

In a wide variety of normal and abnormal conditions no changes in properdin level have been observed. Patients of all age groups, from the newborn* to the elderly, have similar properdin levels, and no sex differences have been encountered. Properdin levels are unrelated to fever or to the leukocyte count of peripheral blood. In patients with infectious diseases, changes in leukocyte count over a range of 100 per cu. mm. to 40,000 per cu. mm. are not associated with changes in properdin level. In patients with various forms of leukemia, normal properdin levels have been observed coincident with leukocyte counts ranging from 600 per cu. mm. to 300,000 per cu. mm. In addition, observations made in conjunction with E. B. Buynak of Georgetown University, Washington, D. C., have indicated that whole blood from patients with extremely low serum-properdin levels has normal phagocytic activity. Other observations indicate that changes in the amounts and ratio of serum proteins have no effect on properdin level. It is not known, however, whether the presence of abnormal serum proteins may affect properdin determination. Some sera from patients with multiple myeloma are anticomplementary, and it is difficult to interpret properdin determinations on such sera. Isoantibodies, heterophile agglutinins, and cold agglutinins do not influence properdin titers.

Data obtained by Benson *et al.*[8] indicate that properdin level is unaffected by anesthesia and uncomplicated surgery. Uncomplicated splenectomy has not been accompanied by change in the properdin level, nor have adrenalectomy

* Serum from infants and children was supplied by R. J. Wedgwood.

and various forms of adrenal substitution therapy caused change in properdin level.[8] In a small group of patients who had received localized radiotherapy to the head, neck, chest, spleen, and back in doses as high as 3,000 r, no change in properdin level was observed.

Certain human diseases are associated with an increased susceptibility to infection. Although it is possible that a predilection to infection might be associated with a spontaneously occurring properdin deficiency, no evidence has been obtained in support of such a view. Disorders of this sort in which normal properdin levels have been obtained during infection-free periods include agammaglobulinemia, chronic recurrent infection in which gamma globulins are normal, bronchiectasis with repeated pulmonary infections, hepatic cirrhosis, acute leukemia, administration of steroids, diabetes and nephrosis. An additional group of patients in whom normal properdin levels have been observed include severely malnourished patients with anorexia nervosa, patients with myocardial infarction, patients with a variety of both localized and metastatic malignancy including both lymphoma and carcinoma, patients with a variety of forms of tuberculosis, and patients with acute and chronic rheumatoid arthritis.*

A number of bacterial infections have been observed in which a change in properdin level has occurred. These include 8 of 15 patients with pneumococcal pneumonia, 14 of 15 patients with pyelonephritis caused by a variety of gram-negative organisms, and individual patients with *Hemophilus influenzae* meningitis, meningococcemia, and bacillary dysentery. In a variety of other infections, including streptococcal pneumonia, primary atypical pneumonia, and subacute bacterial endocarditis caused by *Streptococcus viridans*, normal serum-properdin levels were encountered.

At the time of admission to the hospital, serum-properdin levels of less than 1 unit per ml. were usually observed in those patients who showed a change. Under treatment, the serum-properdin level returned to normal, usually during the first week of hospitalization. No subsequent rise in properdin above normal levels has been detected in this group of patients. Total serum-complement and C'3 levels have been normal, and no change in complement has been demonstrated to be related to the change in properdin level. In 6 of the 8 patients with pneumococcal pneumonia, there was the return to normal properdin level during the first week of hospitalization (FIGURE 1), but in 2 of the patients who were acutely ill on admission and who had protracted courses, the serum-properdin level remained below 1 unit per ml. during the time of the severe illness and returned to normal only during convalescence. The reason for this prolonged low level of properdin is unknown. It is also interesting to note that in the patient with meningococcemia (FIGURE 2) a normal serum-properdin level was detected at the time of admission to the hospital when the organisms were cultured from the blood. On the third hospital day an extremely low properdin level was detected, and thereafter the properdin level returned to normal. The other patients with gram-negative infections had low properdin levels at the time of admission to the hospital.

* Sera from patients with tuberculosis were supplied by Frank Horsfall, and from patients with rheumatoid arthritis by George Heller.

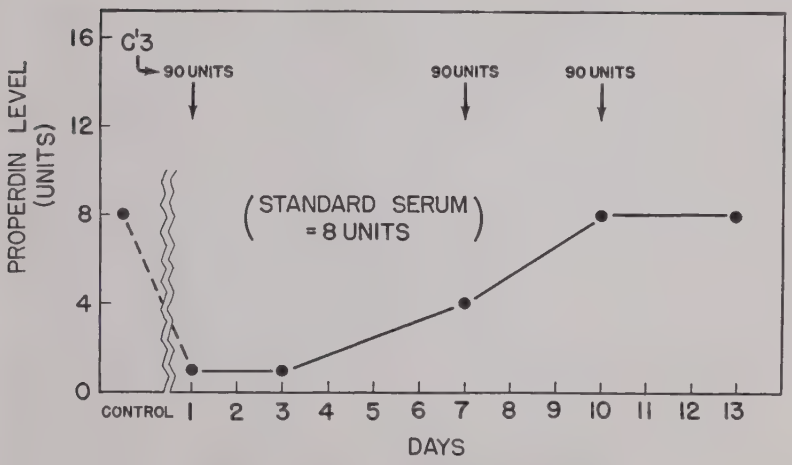

FIGURE. 1. Properdin level in pneumococcal pneumonia.

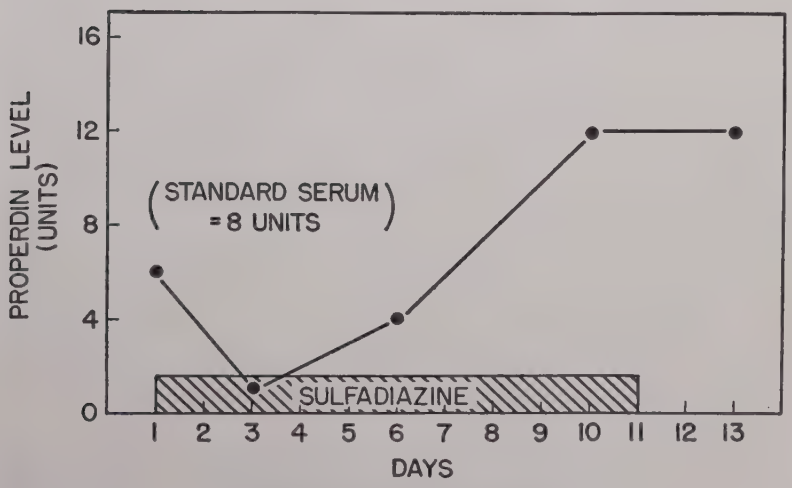

FIGURE 2. Properdin level in meningococcemia.

Observations made in conjunction with E. B. Buynak of Georgetown University on a group of patients with pyelonephritis from a variety of gram-negative organisms indicate that during the acute phase of the infection the serum-properdin level may be extremely low. At the same time the bactericidal activity of the patients' own serum on the organism cultured from the urine is also low. With convalescence there is a return to normal in both the serum-properdin level and the bactericidal activity of the serum, although there is not a

uniform correlation between the level of properdin in the serum and the ability of that serum to kill the infecting organisms *in vitro*.

The mechanism responsible for the fall in properdin level during acute bacterial infection is unknown. The fall during the acute illness may occur as a result of the direct combination of properdin with bacterial products *in vivo*. *In vitro* experiments indicate, however, that amounts of substances such as pneumococcal polysaccharide required for the combination or removal of properdin from serum are far greater than would be present *in vivo* during a pneumococcal infection.[4] There is no assurance, however, that the conditions for the combination *in vitro* are identical to those *in vivo*. It is of interest that low properdin levels have been detected only in those diseases caused by organisms for which there is some evidence for combination of bacterial products with properdin *in vitro*. The fall in properdin may also be due to the combination of the properdin with host-tissue products released as a result of tissue damage during the infection.[9] In numerous other infections presumably involving as much tissue damage as those in which a change in level occurred, however, no change in properdin level has been observed.

Persistently low serum-properdin values have been observed only in patients with paroxysmal nocturnal hemoglobinuria. Although occasional high properdin levels have been detected in some individuals, no disease states characterized by a persistently high level have been observed. Sanford and Landy,[10] however, have recently noted that the administration of extremely small amounts of purified *Eberthella typhosa* lipopolysaccharide resulted in a marked rise in the serum-properdin level. A marked pyrogenic response occurred following injection of 0.1 μg. of the purified lipopolysaccharide in a young adult male. No change in properdin levels occurred for 6 hours after injection, but serum obtained 12 and 24 hours after injection had high properdin levels. Thereafter there was a gradual fall, returning to the initial level at the third day. When the serum samples were centrifuged at 35,000 g. for 2 hours, however, a higher and more sustained rise in properdin level was observed. Thus a striking rise in properdin level was observed following injection of purified bacterial lipopolysaccharide, and an even greater rise was noted when the serum samples were first centrifuged at high speed. This is the only instance in humans in which an abnormally high properdin level has been observed and in which a difference in properdin level between centrifuged and uncentrifuged samples has occurred.

In summary, low properdin levels have occurred in humans with a variety of diseases including paroxysmal nocturnal hemoglobinuria, gram-negative infections, and pneumococcal pneumonia. Elevated levels have been observed after the injection of purified typhoid lipopolysaccharide. The mechanism of these changes remains unknown, but in each instance the clinical state could be correlated with the ability of the responsible agent to interact with the properdin system *in vitro*.

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THE PROPERDIN SYSTEM IN RELATION TO FATAL BACTEREMIA FOLLOWING TOTAL-BODY IRRADIATION OF LABORATORY ANIMALS*

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During the past 2 years studies have been carried out on the properdin system in animals. These studies were a natural outgrowth of observations by Pillemer and his associates[1, 2] demonstrating that the properdin system is responsible, in part, for the bactericidal properties of serum and, secondly, that zymosan combines with properdin *in vitro*.

The present studies are based on 2 hypotheses. First, if the properdin system is, indeed, concerned with humoral mechanisms of host resistance and susceptibility, then the parenteral addition of purified properdin or the active stimulation of properdin production might alter an animal's ability to withstand bacterial infection. Second, if the response of an animal to stimulatory materials or injurious experiences is known, or can be evaluated, then the subsequent ability of the animal to withstand infection might be studied in a predictable fashion.

Total-body irradiation, in the mid-lethal range, 500 r to 800 r, has been used for these studies. In this range it is known that a bacteremia of enteric origin, mainly with *Escherichia coli*, *Pseudomonas pyocyanea* and *Bacillus proteus*, is a frequent terminal event. Total-body irradiation to produce bacteremia has been used with a full understanding that it is, indeed, total-body injury with many auxiliary effects in addition to the infection.

Several other factors complicate evaluation of the role of properdin in infection. Thus many properdin-insensitive strains exist among the gram-negative organisms.[1] Also, it has been noted that while partially purified properdin, freshly isolated, has a sedimentation constant of 24 to 30 S, this material, when stored at 1° C., shows a progressive fall to a sedimentation constant of 3 S with ultimate loss of *in vitro* activity. No information is available yet concerning the interrelations between the sedimentation constant of properdin and its *in vivo* activity. Further, relatively little is known concerning biological differences in properdins isolated from the blood of different animal species. The properdin preparations used in the experiments to be described here have not been characterized as to sedimentation constant or molecular size. In each instance, however, they were assayed by the zymosan method for properdin activity.

Initial interest in the properdin system in infection arose as a result of experiments on tumor production in rats following total-body irradiation. Attempts to increase the number of survivors in such experiments by the administration of antibiotics were under trial when the relationship of the properdin system to the bactericidal properties of serum became known. The present

* This study was supported in part by Grant AT (30-1)-1749 from the Atomic Energy Commission, Washington, D. C.

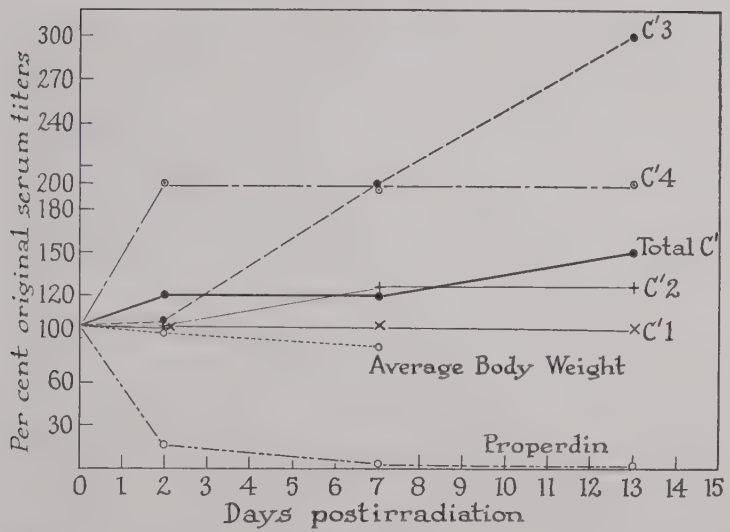

FIGURE 1. Effect on properdin and complement titers in rats following 500 r total-body irradiation.

studies were undertaken to investigate the relationship of properdin to subsequent fatal bacteremia in the serum of irradiated animals.

Initially, a group of rats was subjected to 500 r total-body irradiation. Serum-properdin titers and complement titers were followed progressively for 13 days. A rapid initial fall in serum-properdin titer was observed within the first 48 hours, followed by further fall to undetectable levels (FIGURE 1). Concomitantly, total complement rose to 150 per cent, while C'3 rose to 300 per cent, C'4 rose to 200 per cent, and C'1 and C'2 remained unchanged. A fall in properdin has also been shown in mice and dogs following total-body irradiation.[3]

Following this demonstration of the effect of total-body irradiation on the properdin system, a number of trials were undertaken using limited quantities of partially purified properdin administered parenterally in the postirradiation period. The samples available varied from experiment to experiment. Both human and bovine materials were used, and no characterization other than activity by zymosan assay was available.

In an initial experiment, 3 groups of CF 1, 15 to 18 gm., female mice were subjected to 820 r of total-body irradiation.* One group of 22 mice was injected intravenously on the 2nd, 24th, and 48th hours postirradiation with 50 units of partially purified bovine properdin. The second group of 24 mice was injected intravenously with 0.7 mg. of bovine-serum albumin on the same time schedule. The third group of 24 mice was held as a control. In the properdin-

* Radiation data: 218 kv., 15 ma., 1 mm. Cu + 0.5 mm. Al filter HVL = 1.35 mm. Cu; target distance 32.1 cm.; 128 r per minute at target.

treated group, 14 out of 22 animals survived for at least 16 months. In the albumin and radiation controls there was 100 per cent death in 7 days.

Several experiments were carried out using 600 r total-body irradiation, followed by an intravenous dose of 50 units of properdin administered on selected postirradiation days (TABLE 1). When properdin was administered to mice on the 2nd, 3rd, 4th, or 10th day postirradiation, survival was similar to or less than controls. Animals treated on the 5th day postirradiation, however, showed a fourfold (7/16) greater 30-day survival rate than the untreated 600 r controls (7/64). None of the animals treated on the seventh day postirradiation survived for 30 days (0/16). In addition, an accelerated death rate over the control rate was observed.

These experiments suggest that a fall in the properdin titer of irradiated animals may be an important factor in the development of fatal postirradiation bacteremia. To investigate this relationship more definitively, a large, uniform, well-characterized batch of purified human properdin is needed. This need is emphasized by the results of an experiment employing 4 separate properdins, prepared by 3 different laboratories, that were tested in mice following 600 r on the 24th and 120th hour postirradiation. Fifty units of properdin were administered intravenously at both times in each animal. At 30 days postirradiation these preparations yielded widely variable results from 13 per cent (3/23) to 38 per cent (9/24) survivals as compared to 25 per cent (6/24) for the control group.

As previously reported,[4, 5] intravenous injection of zymosan into CF 1 mice results in an initial marked fall in serum-properdin levels followed by a rebound that is dose-dependent. The properdin response curves following 3 zymosan doses in 15 to 18 gm. CF 1 female mice (5, 25, and 125 mg. per kg.) show initial falls in properdin levels proportional to the dose given. The subsequent rise in titer is inversely proportional to the dose, the smallest dose yielding the most pronounced elevation, while the largest dose results in a return of serum properdin to less than original levels over a 10-day test period. Thus injections of graded doses of zymosan into mice at various time intervals before irradiation provided another approach to evaluation of the role of the properdin system in altering the bacteremia of irradiation.

TABLE 1

SURVIVAL OF CF 1 MICE TREATED INTRAVENOUSLY WITH 50 UNITS OF PARTIALLY PURIFIED BOVINE PROPERDIN FOLLOWING 600 R TOTAL-BODY IRRADIATION

Postirradiation day of treatment	30-day survival
2	1/20
3	3/20
4	2/20
5	7/16
7	1/16
10	0/16
Controls (2, 3, 4 days)	8/24
Controls (5, 7, 10 days)	7/64

Accordingly, the following experiment was performed. Fifteen- to 18-gm. female CF 1 mice of a single shipment were divided into groups of 20. Seven days following receipt of the mice, 0.1 mg. or 2.5 mg. of zymosan per mouse was administered intravenously via the tail vein in a volume of 0.5 ml. The mice were housed, after injection and following 600 r irradiation, in groups of 12 that had received the same zymosan dose. An air-conditioned, humidity-controlled animal room was used, and daily census and weight was recorded for 30 days (TABLE 2).

The results demonstrate a beneficial effect of zymosan on 30-day survival of mice when zymosan is given in low dose (0.1 mg.) at 24 hours before irradiation, or in high dose (2.5 mg.) at 24 or 48 hours before irradiation. In contrast, the low dose (0.1 mg.) administered 48 hours before irradiation not only fails to protect but results in increased susceptibility to irradiation.

In a similar experiment, properdin titers were determined on pooled serum from heart blood in the period immediately prior to and following irradiation of mice that had received 0.1 mg. or 2.5 mg. of zymosan intravenously. Twenty-four hours following the low dose (0.1 mg.), serum-properdin titers were rapidly rising and fell only moderately within 3 hours after radiation exposure (TABLE 3). Forty-eight hours following this dose (0.1 mg.) in a second group of mice, serum-properdin titers were maximally elevated (up to 200 per cent of normal) and showed a marked fall 3 hours after irradiation. At the higher zymosan dose (2.5 mg. per mouse), preirradiation serum titers, although rising, were still below original levels at 24 and 48 hours. Both of these groups showed

TABLE 2

SURVIVAL OF CF 1 MICE SUBJECTED TO 600 R TOTAL-BODY IRRADIATION FOLLOWING INTRAVENOUS ZYMOSAN

Zymosan dose (mg./mouse)	600 r time following zymosan (hours)	30-day survival, S/T
0.1	24	15/24
	48	1/24
2.5	24	16/24
	48	17/24
Control 600 r only	—	7/24

TABLE 3

EFFECT OF INTRAVENOUS ZYMOSAN ON SERUM-PROPERDIN LEVELS OF MICE FOLLOWING 600 R TOTAL-BODY IRRADIATION

Dose (mg.)	Zymosan Time administered before 600 r (hours)	Properdin level		
		Immediately before 600 r	3 hours after 600 r	30-day survival
0.1	24	Rising	Minimal fall	Increased
	48	Maximal	Maximal fall	Decreased
2.5	24	Low and rising	Minimal fall	Increased
	48	Moderate and rising	Moderate fall	Increased
600 r only	—	Falling	Slight fall	—

TABLE 4
SURVIVAL OF CF 1 MICE SUBJECTED TO 600 R TOTAL-BODY IRRADIATION FOLLOWING INTRAVENOUS INJECTION OF MUCIN, DEXTRAN, AND LEVAN

Compound, given intravenously	Dose (mg.)	Time of administration before 600 r (hours)	30-day survival, S/T
Gastric mucin (Hog)	7.5	96	3/25
		24	3/25
Levan B512E	10	96	14/23
		24	3/25
Dextran 1146	10	96	2/25
		24	4/25
Zymosan FL-2	0.2	24	16/25
600 r only	—	—	0/25

only a moderate fall in serum titer 3 hours following irradiation. The 30-day survival data show that a rising properdin titer preirradiation, followed by a small decline postirradiation, is perhaps indicative of protection. Thus it appears that the *level* of the properdin titer at the time of challenge is not correlated with ultimate survival following 600 r total-body irradiation. Rather, the hypothesis is suggested that the *response* of the properdin system to irradiation is altered by pretreatment with zymosan in a manner related both to time of administration and to dosage, and that the survival rate is, in part, dependent on the nature of this response.

In addition to zymosan, a number of high molecular-weight polysaccharides have been studied for their ability to combine with properdin *in vitro*, to alter serum-properdin levels in mice when administered intravenously, and to alter the radiation response when administered prior to irradiation. Of these compounds, Levan B512E satisfies the *in vitro* criteria[6] of removing properdin from serum, inactivating C'3, and binding properdin in such a way that properdin may be eluted from the polysaccharide-properdin complex. Hog gastric mucin and Dextran 1146 fail to meet these criteria fully.

TABLE 4 gives the results of irradiation challenge of mice treated 24 or 96 hours prior to 600 r irradiation. It is noteworthy that both zymosan (FL-2) and Levan B512E yield substantially greater protection than either mucin or Dextran 1146. These experiments demonstrate that certain high molecular-weight polysaccharides will protect against 600 r total-body irradiation. Furthermore, there appears to be good correlation between *in vitro* activity in the properdin system of a given polysaccharide and its *in vivo* activity in protecting against the bacteremia of irradiation.

Summary

Partially purified human and bovine properdin, administered during the postirradiation period, partially protects mice against the bacteremia frequently occurring in this situation. More conclusive data in this area await experimentation with a large, uniform batch of human properdin in the near future. The usefulness of testing this material, however, is heavily dependent

upon further and more precise knowledge of the relationship of molecular size to biological activity and upon the utility of heterologous properdin in mice and other laboratory animals. In addition, the incidence of properdin-insensitive strains of bacteria in a given experiment must also be considered.

Certain high-molecular weight polysaccharides, administered during the preirradiation period, also partially protect mice against total-body irradiation. Such compounds, of which zymosan is the prototype, fulfill certain established criteria of *in vitro* activity in the properdin system.

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THE EFFECT OF IRRADIATION ON NATURAL RESISTANCE TO INFECTION

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Of the various rays and particles that the physicists have developed, the only ones with which we need be concerned are X or gamma rays and fast neutrons. I shall discuss fast neutrons very briefly toward the end of this paper. For our purposes, the effects of X and gamma rays may be considered identical provided they are delivered at approximately the same rate.

In most experimental work, X rays have been used for purely practical reasons. In all of the experiments that I shall describe, the radiation was delivered uniformly to the whole body of the animal and, unless otherwise noted, in a single exposure lasting about a quarter of an hour. The dosage of radiation falls within the range commonly described as moderate, that is, a dose that causes death of 50 to 100 per cent of the animals within 30 days, with the maximum mortality occurring about the 11th or 12th day.

If the dose of radiation is progressively increased, more and more of the animals die earlier, with evidence of injury to the intestinal tract. Bond, Silverman, and Cronkite[1] have called this effect of very high doses the "intestinal syndrome" to differentiate it from the effects of moderate doses designated the "bone-marrow syndrome."

The early, or intestinal, syndrome results from exposure to high doses of radiation, above the LD_{100}. The mean survival-time is short, and, of the pathological findings, damage to the gut is the most prominent. In fact, this syndrome can be produced by irradiation of the gut alone, or of even a portion of it. The intestinal syndrome is a most interesting phenomenon, but one that does not concern us here because death occurs quickly and inevitably. It is mentioned solely for the benefit of those who may not be familiar with the various effects of irradiation.

The bone-marrow syndrome is produced by lower doses of radiation, provided all of the bone marrow is exposed. The mean survival-time is approximately 11 days, and the pathological findings include anemia, hemorrhage, and evidence of generalized infection.

This comparison of the effects of high and moderate doses of radiation explains why the latter are used in the study of host resistance to bacterial infection. This paper is limited to discussing bacterial infection because resistance to virus infections is not much altered by irradiation, at least not in the adult mammalian host.

The effect of irradiation as measured by the 30-day LD_{50} varies considerably among the various species of laboratory animals. Rather than belabor you with the values reported by various workers, I shall merely list the following animals in the order of their decreasing susceptibility: the guinea pig (most susceptible), the dog, mice of various strains, rats of various strains, the hamster, and the rabbit (most resistant). Of these laboratory animals, the mouse

TABLE 1
EFFECTS OF TOTAL-BODY EXPOSURE TO A MID-LETHAL DOSE OF X OR γ RADIATION

	Days postirradiation			
	1–3	4–7	8–14	15–21
Leukopenia	+++	++	+	±
Anemia	−	+	+++	++
Hemorrhage	−	±	++	−
Weight loss	+	±	±	−
Injury to:				
Spleen, lymph glands	++	+++	++	+
Bone marrow	++	+++	++	+
Intestinal mucosa	++	±	−	−

has been the most extensively used, for obvious reasons of economy and convenience.

Total-body irradiation at or near the LD_{50} results in a complex series of physiological disturbances and morphological changes. The tissues of the body vary considerably in their susceptibility to irradiation. Among the most sensitive are the thymus, spleen, lymph glands, bone marrow, and the mucosa of the intestinal tract. At the other extreme is the central nervous system, which is quite resistant.

TABLE 1 lists some of the results of irradiation at or near the LD_{50}. It has been compiled from many sources in an effort to present in very simplified fashion the chain of events that follows exposure to such a dose of X or gamma radiation. Leukopenia develops very rapidly, but the white count begins to rise during the second week. Anemia develops more slowly, as does the tendency to hemorrhage. Weight is lost during the first few days due to loss of appetite and also because of vomiting in those animals that can vomit, for example, the dog and man. Diarrhea may also occur in some animals with resulting loss of fluids and electrolytes. Injury to the intestinal mucosa occurs almost immediately, but is rather rapidly repaired. Injury to the spleen, lymphoid tissue, and bone marrow, that is, to the whole of the hematopoietic system, occurs early.

The time intervals shown in this table are not precise or equal for all mammals, but the time relationships are as accurate as one can make them in a compilation of this sort.

The table reveals the fact that the various manifestations of radiation injury do not occur simultaneously and that the radiation syndrome in this range involves a sequence of events lasting about 3 weeks. It is during the second week postirradiation that susceptibility to infection is most pronounced.

FIGURE 1 shows the results of a series of cultures of heart's blood and spleen of mice sacrificed for this purpose—they are not mice that had died. From the sixth day through the second week there was a high incidence of positive blood cultures.[2]

FIGURE 2 shows the results of similar cultures on mice irradiated with 450 r.

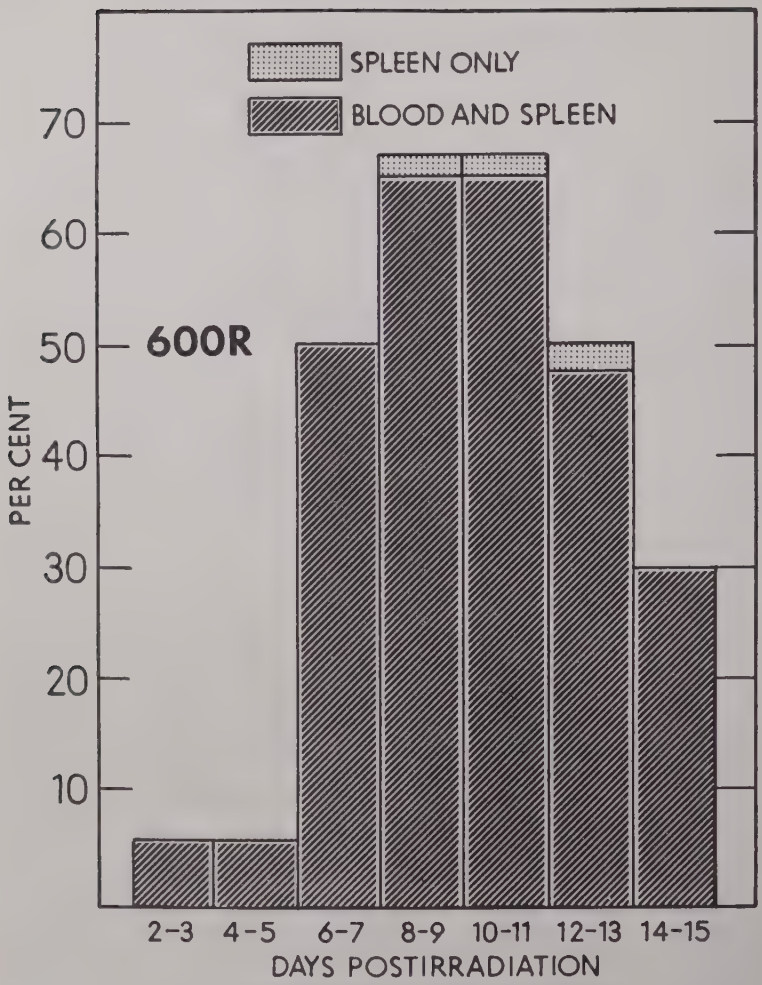

FIGURE 1. Incidence of positive cultures on 288 mice, 20 per day.

In this series the highest incidence of positive cultures occurred during the middle of the second week.

These bacteremias were all caused by microorganisms belonging to the normal enteric flora of this breed of mice, a finding that points to the intestinal tract as the site of origin of these generalized infections.

The severity of the bacteremias is shown in TABLE 2, which summarizes the results on colony counts made from capillary drops of blood. Ninety-one

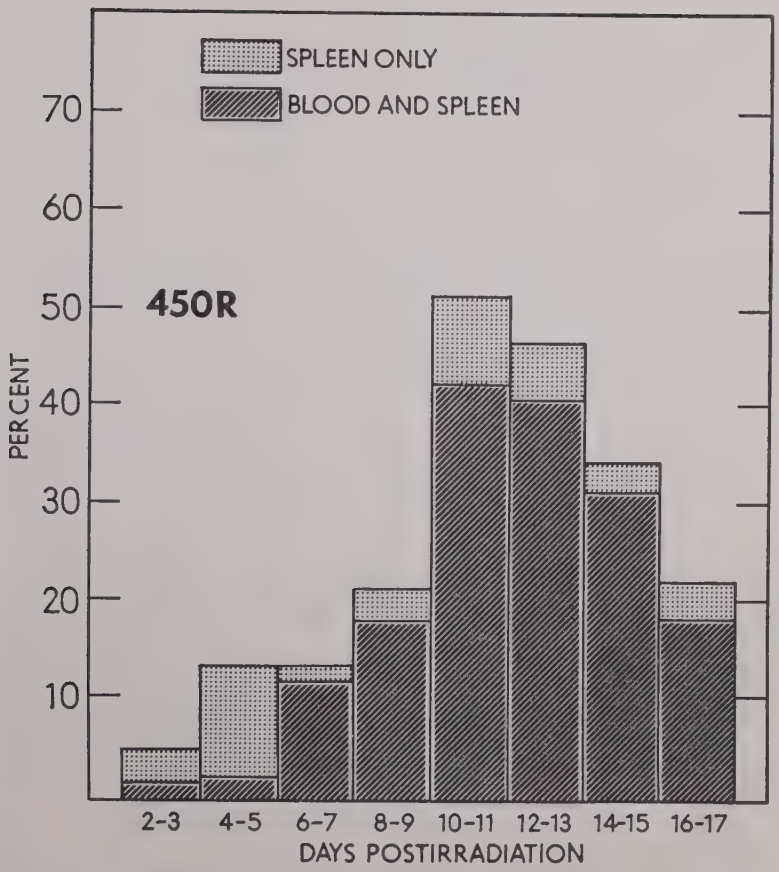

FIGURE 2. Incidence of positive cultures on 597 mice, 35 per day.

per cent of these cultures were pure cultures. The remaining 9 per cent contained only 2 microorganisms.

The duration of life after the onset of bacteremia was found to depend on the species of microorganism that had caused the infection. FIGURE 3 shows the results of an experiment in which each mouse had a drop of blood from the tail cultured each day beginning on the seventh day postirradiation.[3] A plus sign signifies a positive culture in the daily serial cultures and an *X* signifies a positive culture post-mortem. It is apparent that all of the mice with *Pseudomonas* bacteremia died within 24 hours after the first positive culture, most of them within 18 hours. Those with bacteremia caused by *Escherichia coli* or *Paracolobactrum* lived longer, several for more than 4 days with paracoli in their blood.

TABLE 2
SEVERITY OF THE BACTEREMIAS

Colonies per drop of blood	Per cent of positive cultures in	
	600 r series	450 r series
>50	70%	78%
Innumerable	36	18

No mouse with a positive culture survived.

Since *Pseudomonas* was found to be so rapidly fatal once it had invaded the blood stream, it was the microorganism we used for challenge in experiments to determine susceptibility to infection.

A series of experiments was carried out in which irradiated mice were treated with antibiotics to support the thesis that the development of generalized infection plays a significant role as a cause of death from moderate doses of radiation. In other words, these experiments were performed to show that infection actually contributes to and is not merely a result of the lethal process.

FIGURE 4 shows the results of treatment with streptomycin, which was found to be the most effective of the antibiotics used in these experiments. One can see that such treatment resulted in a marked reduction in mortality, which means that the control of generalized infection tided over those individuals that had not suffered irreparable damage until their defense mechanisms had had time to recover from the radiation injury.[4]

It should be pointed out that even in my most successful experiments the mortality rate was never reduced to zero. A certain fraction of the mice always died despite the fact that generalized infection was prevented. It must be presumed, therefore, that this fraction of the mice died of radiation injury per se, and that infection, in their case, was not a determining factor between survival and death.

When I say survival I mean survival for 30 to 60 days, because I am not concerned here with the late effects of radiation injury.

A question that troubled my colleagues and me for a long time was why there is such a long interval between irradiation and the onset of generalized infection. This problem can be more easily understood from the schematic diagram in FIGURE 5.[5] The stippling at the right represents the period during which bacteremias of enteric origin most frequently occurred, as shown in FIGURES 1 and 2. At the lower left is indicated the appearance and disappearance of injury to the intestinal mucosa as described in the histopathological studies of other investigators.[6, 7, 8] Injury to the mucosa of the gut is maximal a few hours after irradiation, but is rapidly repaired.

The curve plotting the leukocyte counts taken from data supplied by Jacobson[9] shows that by the third day the number of circulating leukocytes has fallen below an effective level. There is still an interval of several days before microorganisms from the intestinal tract begin to invade the blood stream. As I have already stated, we were much puzzled by the length of this interval.

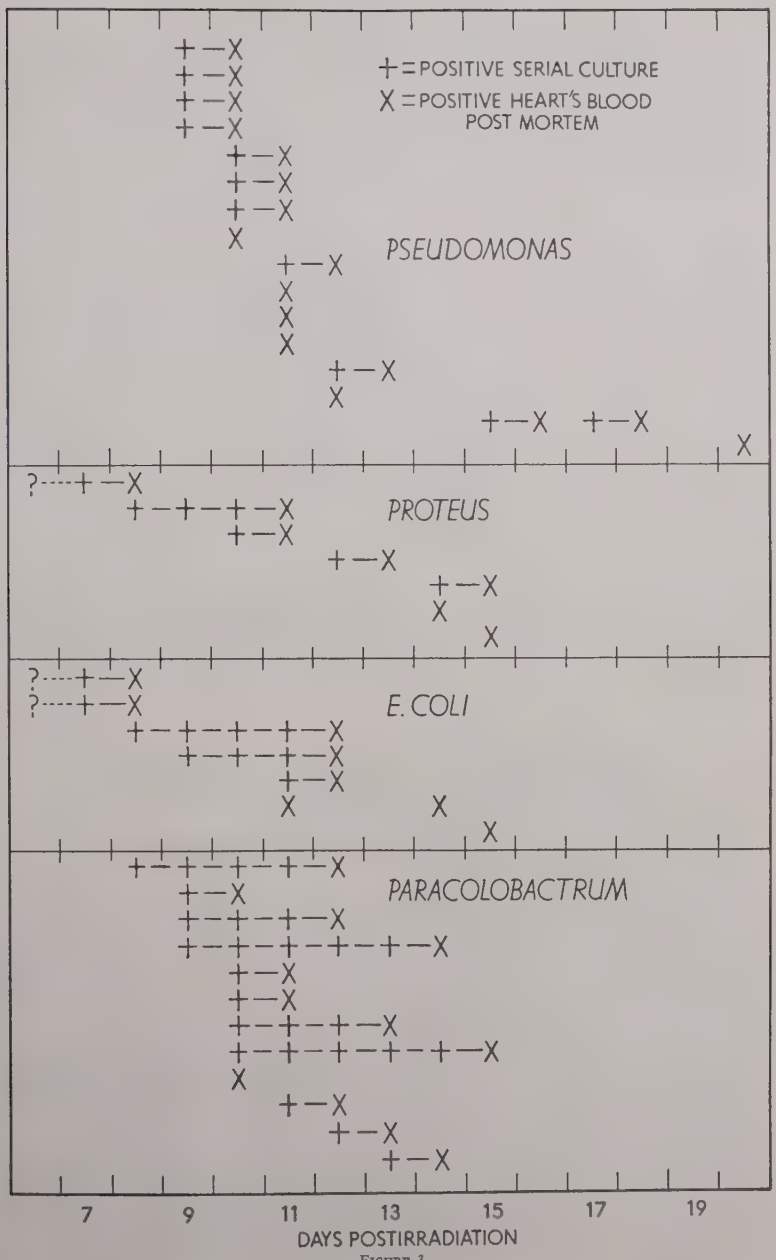
+ = POSITIVE SERIAL CULTURE
X = POSITIVE HEART'S BLOOD POST MORTEM
PSEUDOMONAS
PROTEUS
E. COLI
PARACOLOBACTRUM
7
9
11
13
15
17
19
DAYS POSTIRRADIATION

FIGURE 3.

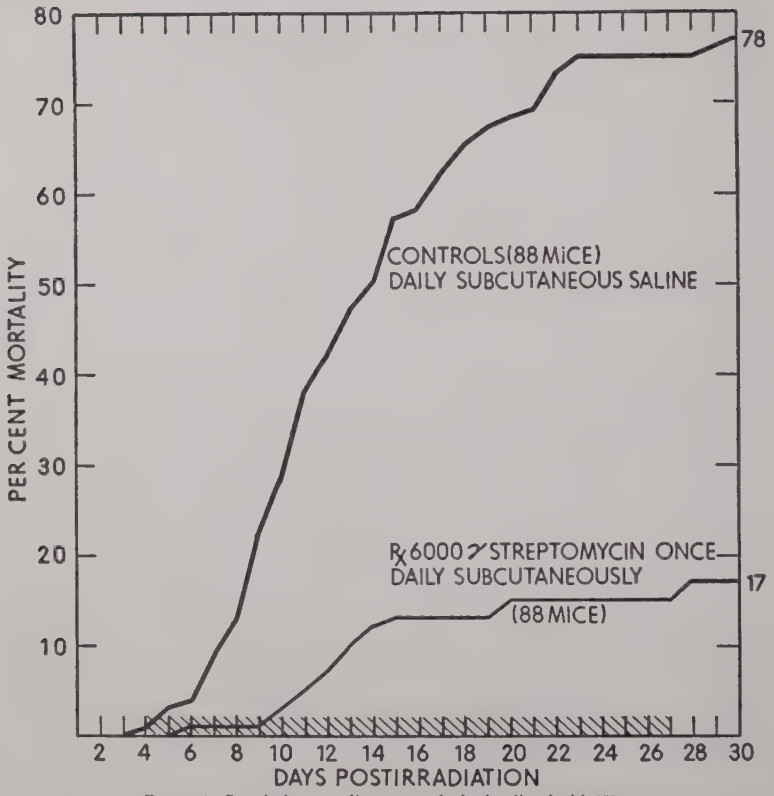

FIGURE 4. Cumulative mortality curves of mice irradiated with 450 r.

The explanation has, I believe, been provided by some experiments of Gordon *et al.*[10] These investigators have shown that the intestinal mucosa of the normal mouse is not impermeable to bacteria, an observation that had been made years ago but that we had to rediscover ourselves. Gordon and his colleagues found that in half of the normal mice examined, intestinal bacteria were present in the mesenteric lymph nodes. The incidence of positive cultures of mesenteric nodes did not increase after irradiation despite the injury to the intestinal mucosa. These investigators did find, however, that a few days after irradiation bacteria began to appear in the liver and spleen before they gained entrance to the blood.

The sequence of events seems to be as follows: in the normal animal, bacteria in small numbers are constantly escaping from the intestinal lumen and are being trapped by the regional lymph nodes and probably by other elements of the lymphoid-macrophage system. By the second or third day postirradiation

the number of circulating leukocytes has fallen to an ineffective level and the lymphoid tissue has undergone marked atrophy. Two important elements in the defense mechanism have, therefore, been eliminated, but the reticuloendothelial system in the liver and spleen continues to function for a few more days and to maintain the sterility of the blood. It is only after the reticuloendothelial system has broken down that bacteria can get through to the blood stream, where they multiply until they cause death.

Additional studies on the functional activity of the reticuloendothelial system were carried out on the rabbit because a larger animal was required for such experiments.[11] Approximately 1 million bacteria (in this case *Klebsiella pneumoniae* type A) were injected intravenously, and samples of blood were withdrawn at intervals thereafter for plate counts—the traditional method of studying the clearance of bacteria from the circulating blood. FIGURE 6 is a composite of results obtained from a number of observations because it was inadvisable to subject an irradiated rabbit to more than a limited number of bleedings. It will be seen that the colony counts on the normal and irradiated rabbits fell rapidly to zero. After a few hours the curves are very different. In the normal rabbits there was a period (indicated by the hump) during which bacteria reappeared in the blood and then disappeared again and the animals survived. In a rabbit that had been irradiated a few days before with 800 r, the numbers of bacteria in the blood increased until the animal died. In other words, the reticuloendothelial system of the irradiated rabbit was able to re-

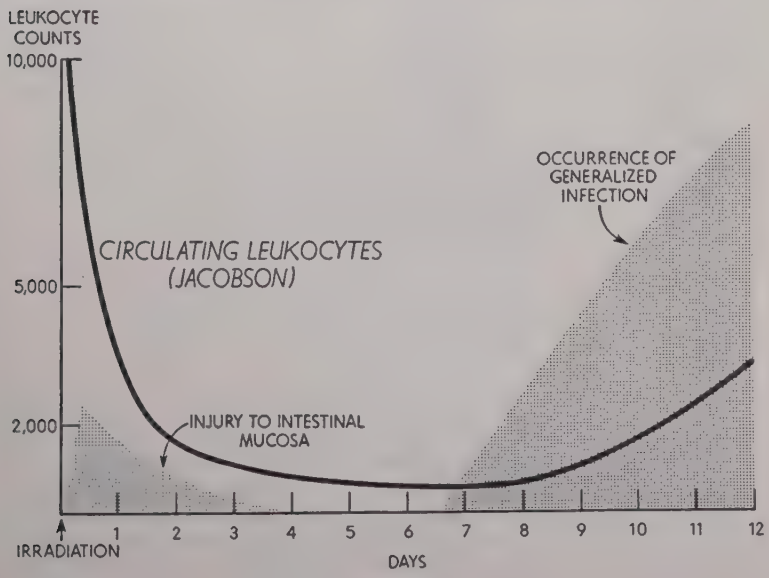

FIGURE 5.

move bacteria from the circulating blood as well as did the reticuloendothelial system of the normal rabbit, but was unable to retain and destroy them. This statement applies to the irradiated rabbit that has been inoculated intravenously with 1 million *Klebsiella*.

While on the subject of rabbits, it should be mentioned that the incidence of naturally occurring bacteremia in irradiated rabbits is considerably lower than in mice.[12] Many of the rabbits with negative blood cultures, however, were found to have intestinal bacteria in their livers or spleens, a result that suggests that their reticuloendothelial system was active in protecting the blood stream from bacterial invasion.

I shall now give brief attention to the effect of fast neutrons. Since death occurs so much more quickly after fast neutrons than after X or gamma irradiation, it was supposed that the survival time after fast neutrons was probably too short to permit generalized infection to develop. It has been found, however, that this is not the case.[13] In mice exposed to fast neutrons, bacteremia does occur, but much sooner than after gamma radiation. Also, the administration of antibiotics has been found to lengthen the fast-neutron survival time and to reduce the mortality, although to a lesser degree than after X or gamma irradiation.[14] It seems, therefore, that after exposure to fast neutrons, infection does play a role as a cause of death, but a less important one than in mice exposed to X or gamma radiation.

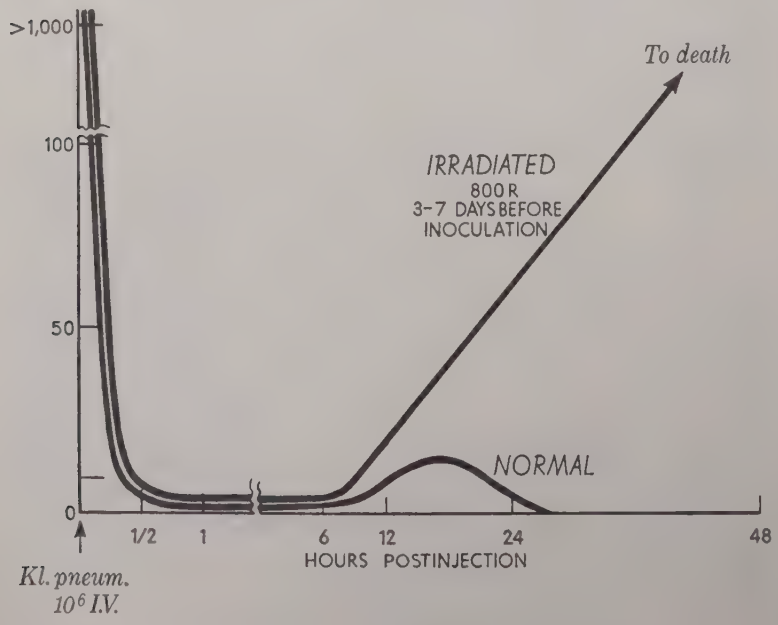

FIGURE 6. Colony counts per ml. of blood.

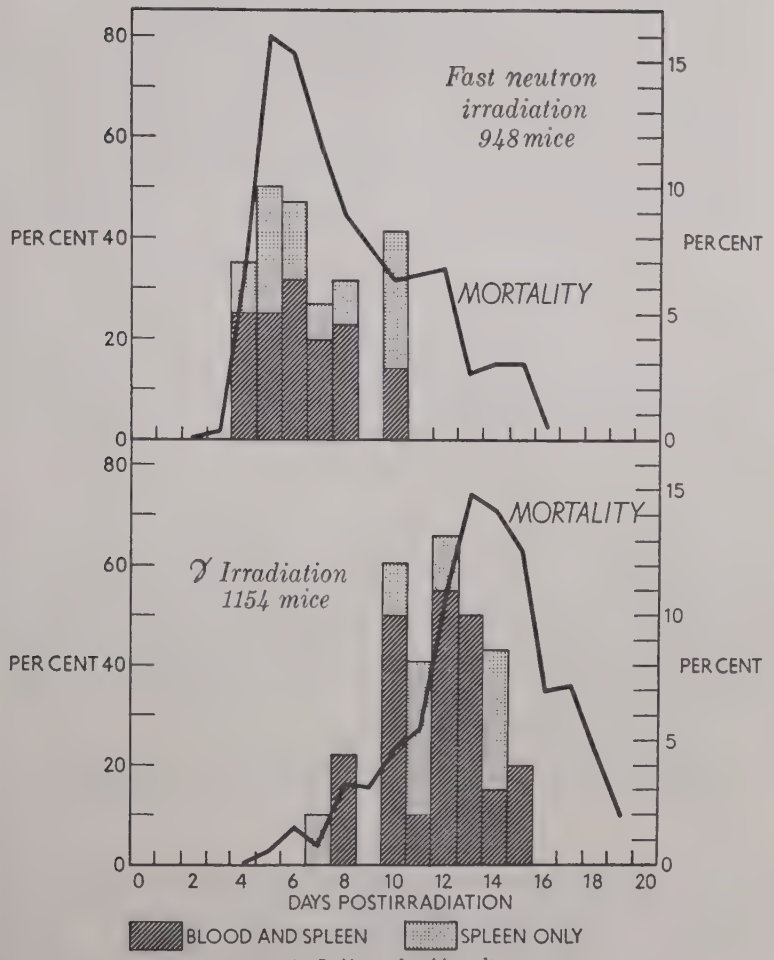

FIGURE 7. Incidence of positive cultures.

Thus far we have been considering the effect of a single brief exposure to radiation. If, however, the dose is spread over a long period of time by exposing animals continuously to low doses of gamma radiation, the effect is quite different. In some preliminary experiments we have found that mice exposed to about 60 r/day can accumulate 2 or 3 thousand r before their resistance to experimental infection is much reduced, despite the severe leukopenia they develop. In the experiment summarized in TABLE 3 the mice began their irradiation at different times so that they could be challenged with the same culture along with unirradiated controls from the same shipment that

TABLE 3

RESULTS OF CHALLENGE WITH *PSEUDOMONAS AERUGINOSA* AFTER CONTINUOUS EXPOSURE TO 63 R PER DAY γ RADIATION

Days exposed	Radiation accumulated	Leukocyte counts (medians)	Deaths following i.p. inoculation with:					
			10^8	10^7	10^6	10^5	10^4	10^3
0	0	10,000	10	0	0	0		
36	2,200 r	1,500	10	9	0	0	0	
43	2,675 r	1,000		10	0	0	0	
50	3,100 r	1,000		10	2	0	0	0
			(10 mice in each group)					

had been set aside for that purpose. Had these mice been irradiated with 600 to 700 r in an acute exposure a week before challenge, they all would have died.[2] These data are presented to bring out the point that postirradiation leukopenia may play a less important role in the mouse's reduced resistance to bacterial infection than we had supposed before we began these studies on the effects of continuous exposure to low doses of radiation.

Summary

I have tried: (1) to outline briefly the more important effects of total-body irradiation on the young adult mammalian host, (2) to indicate the dose range that lowers resistance to bacterial infection, (3) to present a plausible explanation of the pathogenesis of the endogenous bacteremias caused by enteric microorganisms that are entirely harmless for the normal host, (4) to compare the effects of X or gamma rays and fast neutrons and (5) to compare the differences between the effects of a single dose of radiation and prolonged continuous exposure to low-dosage radiation.

I hope I have made the point that ionizing radiation can be profitably exploited in the study of the various factors involved in host resistance to bacterial infection.

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INCREASE IN RESISTANCE FOLLOWING ADMINISTRATION OF BACTERIAL LIPOPOLYSACCHARIDES

By Maurice Landy*

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In the search for a better understanding of natural resistance to infections, a productive approach has been the employment of an ever-increasing variety of methods for altering resistance. Thus adrenal steroids, nitrogen mustard, ionizing radiation, and various dietary regimens have been employed successfully to increase susceptibility. For the most part, the underlying mechanisms responsible for these alterations remain obscure. It is evident, however, that the change is in the host rather than in the parasite. On the other hand, it has long been known that, in addition to the specific antigenic components associated with antibacterial immunity, certain bacterial products may augment nonspecific defense mechanisms of the host.[1, 2] A number of situations have been described that indicate a rapid and transient increase in resistance to experimental infections after the injection of killed bacteria.[3, 4, 5] The identity of the bacterial constituents responsible for this nonspecific immunity is not yet known, nor has the mechanism of this early resistance been clarified other than by the discovery that it is not associated with detectable antibody.

It has recently been shown by D. Rowley[6] that, in mice, cell walls of *Escherichia coli* and *Salmonella typhimurium* evoke this type of resistance to *E. coli* infections, while zymosan, an insoluble carbohydrate derived from cell walls of yeast, produces a similar resistance against a variety of gram-negative pathogens.[7, 8] Moreover, these substances also combine with properdin *in vitro*[9, 10] and alter properdin levels *in vivo*.[8] Present evidence suggests that serum-properdin levels of various animals and their resistance or susceptibility to certain experimental infections may be related. The properdin system,[7] which consists of properdin, complement, and magnesium ions, kills or inactivates a variety of infectious agents *in vitro* and appears to be a factor in natural resistance. These observations suggested to the author that the somatic antigens of gram-negative organisms, that is, the bacterial lipopolysaccharides, might also react with the properdin system and, indeed, might be the *very* component in bacterial cell-wall preparations responsible for the effects described by Rowley. A purified protein-free lipopolysaccharide from *Salmonella typhosa* prepared in our laboratories was tested by Louis Pillemer, who showed that it did, in fact, combine with properdin *in vitro*.[10] This led to a collaborative study with Pillemer on the interaction of lipopolysaccharides with the properdin system. From these investigations it was ascertained that lipopolysaccharides derived from various gram-negative bacterial species give rise, in mice, to an early, nonspecific, transitory resistance to experimental infections with certain enteric pathogens;[11] that these bacterial components produce a considerable rise in the properdin levels of mice;[12] and that, for the most

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part, alterations of the properdin levels thus produced are associated with the degree of resistance to experimental infection.[13] The results of certain phases of the aforementioned collaborative investigation are summarized in this report.

General Considerations

Female white albino mice of the Bagg strain, each weighing 14 to 16 gm., were employed in groups of 10 mice. Pilot experiments indicated that the biological effects produced were essentially identical, whether the lipopolysaccharides were injected intravenously or intraperitoneally. Thereafter injections were made by the more convenient intraperitoneal route. Mice were exsanguinated by bleeding directly from the heart under chloroform anesthesia. Generally 0.5 ml. or more blood was obtained from each animal, and blood from groups of 10 mice was pooled to provide a single serum specimen for properdin assay. Sera were frozen promptly, held at −55° C., coded, and shipped to Pillemer for properdin assay.

Shortly after these studies were initiated it became apparent that the biological changes to be described were markedly affected by the dose of lipopolysaccharide employed and by the time interval between its administration and challenge or between its administration and bleeding for properdin assay. Accordingly, most experiments were conducted as time-dose studies that involved 3 or more dose levels of lipopolysaccharide administered at 7 intervals ranging from 120 to 3 hours prior to infection or bleeding. Consequently each experiment totaled 21 or more groups of 10 mice each. In most instances, the entire experiment was set up in duplicate in order that 1 set of animals could be challenged and the other bled for properdin determination.

In the experimental infection of mice with various gram-negative pathogens, the use of mucin as a host-resistance depressant was avoided since it has been shown that this material is anticomplementary[14] and that it combines with properdin *in vitro* and *in vivo*.[10] Its use would therefore introduce still another factor into an already complex system. Instead, 6-hour agar-grown cultures of challenge organisms suspended in saline served as challenge inocula. The number of organisms administered generally represented 3 to 5 $L.D._{50}$. Unless otherwise indicated, mice were experimentally infected with *Salmonella typhosa* type 2. For a number of years, Bagg mice and the type 2 strain of *S. typhosa* have been used for typhoid studies in these laboratories. This provided a well-standardized experimental infection in which the major immunological components involved have been identified.[15] Thus, in addition to the use of this system in the study of nonspecific resistance, it was also possible to make certain comparisons with specific immunity.

Results

*Screening of bacterial lipopolysaccharides.** The bacterial lipopolysaccharides employed in this study are the somatic antigenic components of gram-negative

* The use of the term "lipopolysaccharide" is not intended to imply that such bacterial products are necessarily homogeneous or composed exclusively of polysaccharide and lipid. For the most part, these substances, as customarily prepared, also contain nitrogenous and other associated materials in varying amounts. Until more is known about which features of chemical structure are essential for each or all of the many biological properties possessed by these complex materials, this terminology should be understood as not implying more than the foregoing.

TABLE 1

BIOLOGICAL ACTIVITIES EXHIBITED BY VARIOUS BACTERIAL LIPOPOLYSACCHARIDES

Product derived from		Prepared by*	Number of preparations tested	Response elicited in mice by 10 μg.	
Organism	Colonial type			Resistance to typhoid challenge	Elevated properdin levels
Escherichia coli (5 strains)	S	D, W, W-L	5	active (5)	active (4)
Hemophilus pertussis	S	W-L	1	active	active
Pseudomonas aeruginosa	S	B, W-L	2	active	active
Salmonella enteritidis	R	W	1	active	negative
Salmonella schottmuelleri	R	W	1	active	active
Salmonella typhosa	S	D, W, W-L	3	active	active
Salmonella typhosa	R	R	1	active	active
Serratia marcescens	S	D, S, W-L	3	active	negative (1)
Shigella flexneri	S	W-L	1	active	negative

* B = Baxter Laboratories, Inc., Morton Grove, Ill.; D = Difco Laboratories, Inc., Detroit, Mich.; R = M. Raynaud; S = M. J. Shear; W = O. Westphal; W-L = M. E. Webster and M. Landy.

organisms. In addition to their specific immunological attributes, they evoke in certain experimental animals and in man a characteristic array of physiological alterations including fever, cellular changes, local skin reactivity, toxicity, and even lethal effects.[16]

A total of 18 lipopolysaccharide preparations, many of which were generously provided by other investigators, were examined for activity in increasing the resistance of mice to infection and for the elevation of properdin levels. As shown in TABLE 1, these products were derived from a total of 6 genera, from a number of serotypes, and from smooth and rough colonial variants. It is noteworthy that they were prepared in 6 different laboratories by varied methods of fractionation and purification. Nevertheless, all were active in producing in mice an increase in resistance to challenge with *Salmonella typhosa*. Many, but not all, caused an elevation in properdin titer of 50 per cent or more above normal. The screening of this collection of preparations for these biological activities was limited to 1 dose level (10 μg.) and 1 time interval (injected 24 hours prior to challenge or bleeding). A more extensive study might well reveal further quantitative, or perhaps even qualitative, differences between these preparations. This survey is sufficient, however, to indicate that the factor responsible for these effects is common to gram-negative organisms and is obtained by a variety of preparative techniques.

Production of host products. It had been observed that following the injection of 1 to 10 μg. of bacterial lipopolysaccharide, properdin titers of mice frequently were elevated but erratic, and failed to show any consistent trend or pattern. It had previously been shown by Pillemer *et al.*[10] that high molecular weight polysaccharides of bacterial origin combine with properdin and inactivate C'3 *in vitro*. The presence of these substances in serum interfered both with properdin and C'3 assays, and these investigators therefore emphasized that high molecular weight polysaccharides first had to be removed by centrifugation at 35,000 g. in order to obtain reliable properdin titers.[10]

A series of experiments was carried out by Landy and Pillemer[12] to ascertain

TABLE 2

PROPERDIN LEVELS OF MICE FOLLOWING INJECTION OF LIPOPOLYSACCHARIDES[12]
Effect of removal of high molecular weight substances

Lipopolysaccharide derived from	Amount injected I.P.	Treatment of serum pools prior to properdin assay	Hours elapsed between injection of lipopolysaccharide and exsanguination						
			3	6	12	24	48	72	120
			Units properdin per ml. of serum*						
	μg.								
Salmonella typhosa 0901	10	None	24	9	12	15	24	24	24
		Centrifuged†	12	18	30	36	36	36	24
Escherichia coli 08	10	None	24	24	12	12	24	30	30
		Centrifuged†	12	24	30	30	30	36	36
————	0.5 ml. of saline	None				12			
		Centrifuged†				12			

* Groups of 100 mice were employed to obtain the serum pools representing each product-time interval.
† Thirty-five thousand g. for 2 hours at 2° C.

whether the removal of high molecular weight substances from serum obtained from mice receiving small amounts of bacterial lipopolysaccharides would influence their properdin titers. Properdin titrations on such mouse sera before and after centrifugation at 35,000 g. for 2 hours are shown in TABLE 2 It will be seen that between 6 hours and approximately 3 days following administration of lipopolysaccharide, the untreated sera of these mice show lower and, at times, erratic properdin values. On the other hand, after centrifugation the same sera present a consistent pattern of early rise in properdin levels, reaching a maximum of approximately 3 times the normal, which is maintained for several days. Thus 6 to 12 hours after injection of this quantity of lipopolysaccharide into mice, high molecular weight substances appear in the blood that interfere with the inactivation of C′3 by zymosan. These substances persist in the circulation for 48 to 72 hours. The properdin levels are then no longer affected by centrifugation. The appearance of these substances in the blood shortly after injection of lipopolysaccharide suggests that they are either elaborated or released by the host following stimulation or injury by lipopolysaccharide. The nature of these host products is presently being investigated. Preliminary evidence suggests that they are protein-polysaccharide-lipid complexes. Lipopolysaccharides previously had been shown to combine with properdin *in vitro*,[10] but the quantities required for such combination are of a completely different order of magnitude, inasmuch as the *in vitro* interactions require 100 to 200 times the amount necessary for this *in vivo* effect. These results therefore suggest that, following injection of lipopolysaccharide, substances of endogenous origin appear in the blood, and their presence interferes with the assay of properdin. All subsequent properdin titrations were done on centrifuged serum samples.

Effect on resistance to various experimental infections. For reasons previously stated, most of these studies were carried out with the experimental infection

produced in mice by the intraperitoneal inoculation of *Salmonella typhosa*. It was of interest, however, to determine to what extent the observed increased resistance to infection produced in mice by lipopolysaccharide was also applicable to other experimental infections. These infections fell into 2 major categories, those produced by gram-negative pathogens and those initiated by gram-positive bacteria. Among the former, the organisms examined in addition to *S. typhosa* type 2, were *Escherichia coli* strain E-2 380 V (Rowley), *Pseudomonas aeruginosa*, and *Proteus vulgaris* (these 2 cultures were isolated from irradiated mice with bacteremia by W. W. Smith). The $L.D._{50}$ for these cultures ranged from 10 to 50 million organisms, and all were administered in saline at a level of approximately 1 to 3 lethal doses. These comparisons were made in the form of time-dose experiments in which groups of mice received either 10, 1, or 0.1 μg. of *S. typhosa* lipopolysaccharide at various intervals prior to infection with the 4 challenge organisms. The mortality data obtained are too voluminous and too complex to be presented here. That portion of the data given in TABLE 3, however, serves to indicate the nature of the differences in the pattern of resistance to these infective agents. In this portion of the study the mice received 1 μg. of lipopolysaccharide at the indicated intervals prior to infection with the 4 challenge organisms.

It is apparent that the effect of this treatment of mice with a given dose of lipopolysaccharide varies considerably for the 4 experimental infections, particularly as concerns the rate of development of resistance and its duration. Not shown, but also exerting an important effect, is the quantity of lipopolysaccharide administered. The reasons for these differences remain to be determined. The differences may be related, however, to any of a number of factors, such as the rate of multiplication of the challenge organism, quantitative differences in their susceptibility to the bactericidal action of the properdin system, or their relative avidity in combining with properdin and depleting the host's reserves of this component.

Treatment of mice with bacterial lipopolysaccharides in a manner shown to be highly effective in protecting against experimental infection with the aforementioned gram-negative bacterial species failed to produce a demonstrable effect on the susceptibility of these animals to challenge with *Staphylococcus*

TABLE 3

INCREASE IN RESISTANCE OF MICE TO INFECTION WITH VARIOUS GRAM-NEGATIVE PATHOGENS FOLLOWING ADMINISTRATION OF A BACTERIAL LIPOPOLYSACCHARIDE

Challenge			Hours elapsed between injection of 1 μg. *Salmonella typhosa* lipopolysaccharide and challenge					
Organism	Number	$L.D._{50}$'s	6	12	24	48	72	120
			Per cent survivors					
S. typhosa	5×10^7	5	100	90	20	10	0	0
E. coli	5×10^7	3	100	100	100	100	70	10
Ps. aeruginosa	8×10^7	3	30	70	70	60	50	50
P. vulgaris	1×10^8	2	60	70	90	70	40	10

aureus, *Streptococcus pyogenes* group A, or *Diplococcus pneumoniae* type 1. In each instance the challenge employed was minimal in amount in order to provide the most favorable conditions for demonstrating even a slight increase in resistance by the injection of lipopolysaccharide. These animals, however, succumbed to each of these experimental gram-positive infections in a manner indistinguishable from the controls.

It should be pointed out that these 3 gram-positive cocci are insensitive to the properdin system as measured by the *in vitro* bactericidal test. On the other hand, the 4 gram-negative organisms exhibit a high degree of susceptibility to the properdin system.

Time and dose relationships. Early in these studies it was observed that increased resistance to challenge with *Salmonella typhosa*, and with certain other gram-negative pathogens, developed in mice within hours after injection of a bacterial lipopolysaccharide. It soon became apparent, however, that the induction of this early rise in resistance and the accompanying increase in properdin titer were affected by a number of factors. Prominent among these were: the lipopolysaccharide preparation employed, that is, the bacterial source and/or mode of preparation; the quantity administered; the interval between its injection and challenge, or drawing of blood for properdin assay; the challenge organism; and the size of the challenge inoculum. Other less critical but nevertheless important variables were the strain of mice employed and a number of factors pertaining to the challenge inoculum, such as age of the culture and menstruum used for suspending the challenge.

It was not possible to examine systematically all of these factors. It was established, however, that given an effective lipopolysaccharide and an appropriate experimental infection, the major variables affecting properdin titers and survival were the dose of lipopolysaccharide administered and the interval between its injection and challenge or between its injection and bleeding for properdin assay. These observations, made in a series of pilot experiments, led to the adoption of "dose-time" experiments as a means of obtaining more complete information on the effect of lipopolysaccharide on properdin titers and on resistance or susceptibility to infection.

The administration of a *large* dose (100 μg.) was attended by a considerable delay, of 12 hours or more, in the development of resistance. During this time the animals were *more* susceptible to challenge than were normal mice. With smaller quantities (10, 1, or 0.1 μg.) delay in the development of resistance was reduced to a few hours and, during this induction phase, there was no evidence of increased susceptibility. With respect to the duration of the effect, the converse occurred, and the 100 μg. quantity provided the most prolonged period (5 days) of increased resistance, 10 μg. 1 day, 1 μg. approximately 12 hours, and 0.1 μg. 6 to 8 hours.

Mice similarly treated and bled for properdin assay, at the time the aforementioned groups were challenged, showed the following properdin responses:

The injection of 100 μg. caused a fall in properdin titer to approximately one half of normal in a period of between 12 and 24 hours. This fall was succeeded by a marked rise to 2 to 3 times normal and persisted for 5 days.

The injection of 10 μg. resulted in an increase as early as 6 hours. The level

then rose to 2 to 3 times normal at 24 hours and persisted for an additional 48 hours. Attention should be called to the fact that this elevation occurred without the prior fall in properdin titers observed when large doses of zymosan are employed. This effect may, therefore, be due to a direct stimulation of the synthesis or release of properdin rather than to a mechanism that represents over-compensation, or "rebound," on the part of the host.[12]

The effect of 1 μg. was qualitatively similar to that obtained with 10 μg., except that the increase in properdin titer was somewhat less. No alteration in properdin levels occurred following either 0.1 μg. of lipopolysaccharide or 0.5 ml. of saline. Indeed, the constancy of the values in control animals and those receiving 0.1 μg. was noteworthy. Thus, with regard to both the development of resistance and the rise in properdin levels, the rate, magnitude, and duration of these effects were all related to the dose of lipopolysaccharide employed.

On the whole, some sort of relationship was seen between the elevated properdin titers and the degree of protection developed in response to injection of lipopolysaccharide. In seeking to relate increased protection to elevated properdin titers, however, it was apparent that there was a number of serious inconsistencies in such a proposed relationship. For example: (1) properdin titers generally rose after resistance had developed; (2) in mice receiving 100 μg. of lipopolysaccharide the rise in resistance occurred 12 hours after injection, a time when properdin levels were at their very lowest; (3) elevated properdin levels generally persisted well after resistance had waned; and (4) increased resistance for a period of 4 to 8 hours was afforded by as little as 0.1 μg. in the absence of any demonstrable increase in properdin titer.

Influence of infection on properdin response. It was apparent that, following the administration of lipopolysaccharide, the ensuing pattern of elevated properdin levels and increased resistance did not always run parallel. That is to say, the properdin levels of the animal prior to infection did not uniformly reflect the ability of the host to maintain that titer during infection. It was visualized that, following the initiation of infection, host-parasite interactions become extremely complex and that, during this period, properdin levels might differ considerably from those of the prechallenge pattern. In order to obtain information on the nature of these changes, the bacteriologic sequence of events and the serum-properdin alterations were followed postchallenge in specifically and nonspecifically protected mice, as well as in controls. For specific immunization, mice were given 1 μg. of purified Vi antigen 5 days prior to challenge. This treatment affords a complete protection that is accounted for by the specific antibody produced in response to this antigenic stimulus.[17] Nonspecific protection was provided by injection of 10 μg. of a *coli* lipopolysaccharide. This effect is independent of the production of specific antibody. Previous tests have shown that both types of treatment provide complete protection against challenge with *Salmonella typhosa*.

In control mice, properdin titers remained constant during the period of 3 to 6 hours postchallenge. By 12 hours, when deaths began to occur, however, there was a drop in properdin to two thirds of normal that in time became even more pronounced (one third of normal) as an increasing proportion of the

animals became moribund. These changes were correlated with increasing bacterial populations in these animals, as determined by viable count of peritoneal contents, rising from 270 million at 3 hours to 412 million at 6 hours, 4 billion at 12 hours, and 20 billion at 24 hours, when the last of the controls succumbed. On the other hand, mice treated with 10 μg. of a *coli* lipopolysaccharide 24 hours earlier and exhibiting peak properdin levels at the time of challenge showed a contrasting pattern of bacteriologic and properdin responses. During the first 6 hours there was moderate multiplication of organisms from the 50 million inoculated to 300 million, and a drop in properdin titer from the initial high value, approximately twice normal, back to normal. During the period from 12 to 18 hours postchallenge, the very time when, in the controls, bacterial populations were rapidly rising to lethal levels and properdin reserves were being depleted, the lipopolysaccharide-treated mice not only were maintaining normal properdin values, but these were increased, in fact, within 12 hours to 3½ times normal, a value extremely high for this species. At the same time, further bacterial multiplication was suppressed and the number of organisms declined from 102 million at 12 hours, to 4 million at 18 hours, and 0.4 million at 24 hours. These are well below lethal levels. Thus, at the time the control mice were dying rapidly, the properdin titer of the lipopolysaccharide-treated animals was at least 7 times higher than in the controls, and these animals survived.

The Vi-immunized mice present an entirely different type of response. In the presence of specific antibody, the multiplication of organisms ceased promptly and the numbers of viable bacilli fell precipitously from 110 million at 1 hour postchallenge, to approximately 40,000 at 6 hours, and all animals survived. It is especially noteworthy that in these mice the properdin levels remained quite unchanged.

Relationship of timing and dose of lipopolysaccharide to postchallenge properdin alterations. The initial postchallenge experiment provided some insight into the nature of the properdin alterations following the initiation of infection and suggested that this experimental approach might help to resolve the discrepancies, previously pointed out, in the relationship of properdin levels in normal mice to their subsequent resistance or susceptibility to infection. Of these, the 3 most divergent situations were tested, as follows:

(1) In mice receiving 100 μg. there occurred a rise in resistance at 12 hours, becoming complete at 24 hours, the interval when properdin levels are at their very lowest. Accordingly, groups of mice were injected with 100 μg. of lipopolysaccharide at intervals of 24, 12, and 6 hours prior to challenge. At 3, 6, 12, 18, and 24 hours postchallenge, groups of mice representing treatment at the 3 time intervals tested were bled for properdin assay, and mortality was determined in mice similarly treated. Mice injected 6 hours prior to challenge were not protected. In these animals there occurred a continuous decline in properdin titers from the normal to one third of this value and death. Of the mice injected 12 hours prior to challenge 70 per cent were protected. In such animals the properdin level was one third of normal at the time of challenge and remained at this low level during the first 6 hours postchallenge, rising to two thirds at 12 hours and maintained through 24 hours, then rising to above

normal at 48 hours in the survivors. The mice injected 24 hours before challenge all survived. At the time of challenge, the properdin level was two thirds of normal in animals so treated and remained at this value during the first 12 hours postchallenge, rose to normal at 18 hours, rose above normal at 24 hours, and to twice normal at 48 hours postchallenge.

(2) The injection of 10 μg. of lipopolysaccharide resulted in essentially complete protection when given at 6, 12, or 24 hours prior to challenge, and little or no protection when the interval between its administration and challenge with *Salmonella typhosa* was increased to 48 or 72 hours. Groups of mice were injected with 10 μg. of lipopolysaccharide at 72, 48, 24, 12, and 6 hours prior to challenge. The lack of protection in mice injected 48 or 72 hours earlier was manifest even though properdin levels in such mice were 2 to 3 times the normal value. Thus, under the impact of the bacterial challenge, properdin levels in such mice declined precipitously to one third or even one sixth of the normal level at the time of the demise of the remaining animals. On the other hand, mice treated at the 24-hour interval were just approaching peak properdin titer at the time of challenge. In animals treated in this manner, the challenge caused a rapid drop from the elevated properdin values down to the normal range, and this was then succeeded by a swift and steep elevation of properdin to very high titers. These animals survived. Treatment at 12 hours prechallenge produced a modest rise in properdin while, at the 6-hour interval in this experiment, there was no discernible increase in the level of properdin. Following challenge, however, such animals manifested a continued and progressive rise in properdin titers that at 24 hours postchallenge were approximately 3 times as high as those of normal untreated mice.

(3) In those mice receiving 0.1 μg. of lipopolysaccharide there was observed a rise in resistance to challenge between 4 and 8 hours after injection in the absence of any demonstrable rise in the properdin level. Groups of mice were therefore injected with 0.1 μg. of lipopolysaccharide 12 and 6 hours prior to challenge, and their properdin levels followed after challenge. The mice receiving lipopolysaccharide 12 hours before challenge succumbed, while those treated 6 hours before challenge survived. In the former, the normal properdin value at the time of challenge remained at this level during the first 6 hours, declined to two thirds of normal at 12 hours, and to one third of normal at 18 hours, after which all animals succumbed. The mice injected 6 hours before challenge all survived. In such animals the initial normal value, under the impact of challenge, fell to two thirds within 3 hours, briefly dipped to one third of normal, again rose to two thirds at 24 hours and, by 48 hours had returned to normal.

In contrast to the treated animals, control mice exhibited little or no alteration in properdin levels during the first 6 hours postchallenge, but at 12 hours these had fallen to one third of normal and, at 24 hours, the remaining moribund animals exhibited only one sixth the properdin titer present initially.

These experiments show that mice may be resistant to infection despite normal or even subnormal properdin titers as a result of treatment with either high or very low doses of lipopolysaccharide at certain intervals prior to challenge. The data obtained indicate that in such animals there generally occurs

little or no rise in properdin titers following challenge. Instead, properdin titers are maintained at either normal or only moderately reduced levels, in contrast to a progressive decline to exceedingly low levels and death in control mice. Taken as a whole, these observations suggest that, in themselves, properdin levels determined prior to challenge may not be a reliable guide or index to the animal's reaction to infection. Rather, the critical measure of the effectiveness of the treatment employed is its effect in enabling the animal to maintain either normal or elevated properdin levels during the early phases of infection. Obviously, individual properdin values do not provide this information.

Discussion

This investigation had as its primary objective the determination of the effect of bacterial lipopolysaccharides on resistance of mice to certain infections. It was readily shown that following injection of a single small dose of lipopolysaccharide there occurred an early increase in resistance to gram-negative pathogens and that, in a general way, this increased resistance was associated with either elevated properdin levels or the ability of mice to maintain normal properdin levels, in contrast to control animals that die when their properdin reserves are depleted. The changes in properdin that, in a general way, appear to reflect the resistance or susceptibility of mice to experimental infection have been stressed, inasmuch as it is now known that this serum component, together with complement and magnesium, probably represents the humoral bactericidal system of the host. Wardlaw and Pillemer[18] have reported that relatively small changes in properdin levels *in vitro* suffice to bring about major changes in the rate and extent of destruction of gram-negative organisms in bactericidal tests, suggesting that even moderate elevation in properdin titer may prove important.

It is recognized that the host attribute referred to as natural resistance is complex and is the sum total of an undetermined number of factors of which properdin, however important it may be, is but one. Moreover, it is also appreciated that the parenteral administration of lipopolysaccharide initiates in the host a complex series of reactions, of which probably only a few have thus far been identified. Thus, it may be that these other alterations in the host, such as leukocytic changes and increase in adrenal output, produced by lipopolysaccharide, also participate in the general effects on resistance as here described. Of the known alterations produced in the host by bacterial lipopolysaccharides, however, the effects on properdin at present appear to provide the most significant correlation with the observed changes in natural resistance to certain experimental infections.

Summary

It has been shown that injection of bacterial lipopolysaccharides derived from a variety of gram-negative bacterial species evoke in mice a rapidly developing rise in resistance to infection with gram-negative pathogens. This is accompanied by an elevation in properdin titer to levels 2 to 3 times the normal. The

rate, magnitude, and duration of both responses are related to the quantity and timing of the lipopolysaccharide administered.

High molecular weight substances appear in the blood at the time properdin levels are elevated. These substances, which appear to be protein-polysaccharide-lipid complexes of endogenous origin, presumably represent the result of injury to or stimulation of the host by bacterial lipopolysaccharides.

The increased resistance to infection evoked in mice by bacterial lipopolysaccharides appears to be effective only against infections produced by endotoxin-bearing organisms. These gram-negative bacterial species are highly susceptible to the bactericidal action of the properdin system.

Properdin levels in normal animals, just prior to infection, give an incomplete picture of the reaction of the host to the infective agent. In mice experimentally infected with gram-negative pathogens, however, properdin levels determined at various intervals postchallenge do reflect the course and outcome of the infection. Thus in control animals properdin titers progressively decline until the host reserves are depleted and the animals die while, in mice appropriately treated with lipopolysaccharide, properdin levels are either maintained in the normal range or greatly increased, depending on dose and time of administration of lipopolysaccharide, and this is accompanied by successful management of the infection. The fate of the host apparently is determined by the degree to which it has been "conditioned" by lipopolysaccharide, that is, the rapidity and extent to which properdin subsequently is mobilized and maintained in response to the stimulus of infection.

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SOME FACTORS AFFECTING THE RESISTANCE OF ANIMALS TO BACTERIAL INFECTION

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Since the demonstration by R. F. J. Pfeiffer in 1894 of the lysis of cholera vibrios in the guinea pig peritoneum, we have gradually come to believe that the bactericidal powers of the body fluids play an important role in defense against microbial invasion. This anitmicrobial activity is seen best in serum and is most effective against the gram-negative organisms. The nature of this bactericidal activity is known to be extremely complex. Four components of complement, magnesium ions, and now properdin (Wardlaw and Pillemer, 1956) are known to be essential reactants. On the other hand, nothing appears to be known about the bacterial reactant or substrate for this reaction. This latter factor has been the initial aim of the work of my associates and myself.

The presumptive test for bacterial substrate that we have used is the ability of a preparation to compete with living cells for the bactericidal serum system, the result being a delay or inhibition of the rate of killing of the living bacteria (FIGURE 1). In our attempts to isolate the substrate in pure form we have been guided by the observation that rough mutants of *Escherichia coli* or *Salmonella paratyphi* are killed by serum much more rapidly than the smooth strains from which they are derived (Rowley, 1956), as shown in FIGURE 2. It seemed that this might be a factor of the endotoxin content of the organisms, and we subsequently showed that the lipopolysaccharide fraction of the endotoxin gives the substrate test above.

The other fractions not containing lipopolysaccharide obtained in the phenol/water extraction method of O. Westphal were much less active in the presumptive substrate test.

The early changes in immunity of mice to *E. coli* (Rowley, 1955) or to *S. typhi* (Field, Howard, and Whitby, 1955) infections consequent to the intravenous injection of bacterial cell walls can all be produced by lipopolysaccharides in a minimum dose of about 1 μg./20 gm. mouse (TABLE 1). It has been suggested (Rowley, 1955) that these early changes in immunity can be accounted for by the neutralization of the serum bactericidal reaction by the bacterial reactant, followed within 24 to 48 hours by a compensatory increase in the amount and activity of the bactericidal mechanism. By this hypothesis the lipopolysaccharide would contain the bacterial substrate within its very large molecule.

At this stage let me summarize the evidence that implicates lipopolysaccharide as a bacterial substrate:

(1) Lipopolysaccharide inhibits bactericidal action of serum *in vitro*; that is, it may be competing with the substrate present in the living bacteria.

(2) Avirulent strains of *E. coli* contain less lipopolysaccharide and are more sensitive than smooth strains of *E. coli* to killing by serum (Rowley, 1954 and 1956).

(3) Injection of small amounts of lipopolysaccharide into guinea pigs or rabbits temporarily decreases and then increases the bactericidal power of their sera (TABLE 2).

(4) Concurrently with these changes there is a temporary decrease followed by an increase in immunity to various infections.

We may then ask: what is the nature of the reaction between serum and lipopolysaccharide if the latter is the bacterial substrate?

To investigate this reaction we have prepared the lipopolysaccharide from *E. coli* 2380 labeled with P^{32} by growing the organism on a minimal medium restricted in phosphorus, to which 10 mcurie of P^{32}/liter had been added (Rowley, Howard, and Jenkin, 1956). The bacteria were washed twice and disrupted in a Mullard ultrasonic generator. The deposit from centrifugation at 30,000 g. was then extracted with phenol/water by the procedure of Westphal, Luderitz, and Bister, 1952. The water layer was dialyzed to remove phenol, and was precipitated with 8 volumes of ethyl alcohol in the presence of sodium acetate. The sediment was redissolved in water and adjusted to pH 4 to precipitate a small amount of nucleic acids. The supernatant layer was readjusted to pH 7 and dialyzed, and the fraction sedimenting between 10,000 and

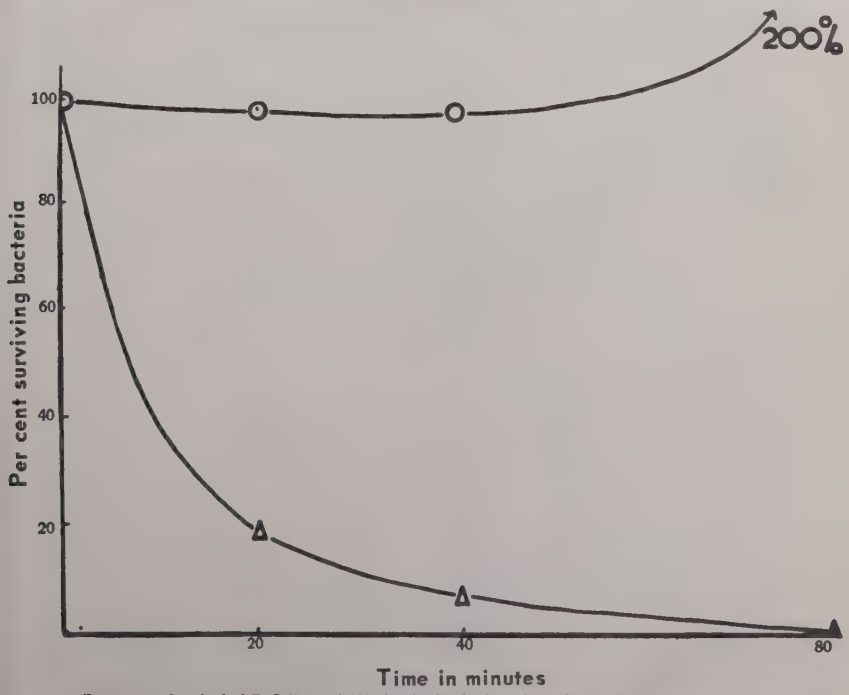

FIGURE 1. Survival of *E. Coli* 2206 during incubation in the guinea pig serum. △ = alone; ○ = together with 0.1 mg./ml. *E. coli* 2380 cell walls.

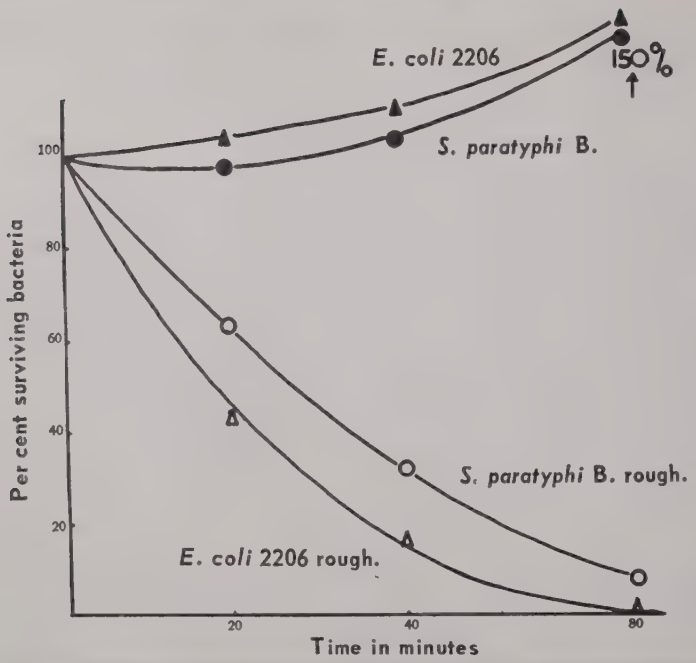

FIGURE 2. Survival of smooth strains and their rough mutants at 37° C. in guinea pig serum diluted 1:4 with minimal medium.

TABLE 1

THE SUSCEPTIBILITY OF MICE TO CHALLENGE BY *E. COLI* 145, 48 HOURS AFTER THE INTRAVENOUS INJECTION OF PURIFIED LIPOPOLYSACCHARIDE FROM *E. COLI* 2380

Dose of lipopolysaccharide injected	Challenge dose of *E. coli* 145			Percentage deaths
	10^6	10^5	10^4	
0.05 mg.	4/21	1/21	1/21	9
0.01 mg.	15/21	6/21	2/21	36
0.001 mg.	10/15	5/15	4/15	42
Controls	15/15	11/15	13/15	86

TABLE 2

EFFECT OF INJECTING 1 MG. CRUDE LIPOPOLYSACCHARIDE FROM *E. COLI* 2380 INTRAVENOUSLY INTO EACH OF 4 GUINEA PIGS, ON THE SUBSEQUENT BACTERICIDAL POWER OF THE POOLED SERUM

Time after injection of 1 mg. crude lipopolysaccharide	Serum dilution producing 50% bacterial killing in 80 mins.	Per cent bacterial survival in dilution 1:4 after 80 mins.
Start	1:4	50%
1 hour	Undiluted	100%
24 hours	1:16	10%

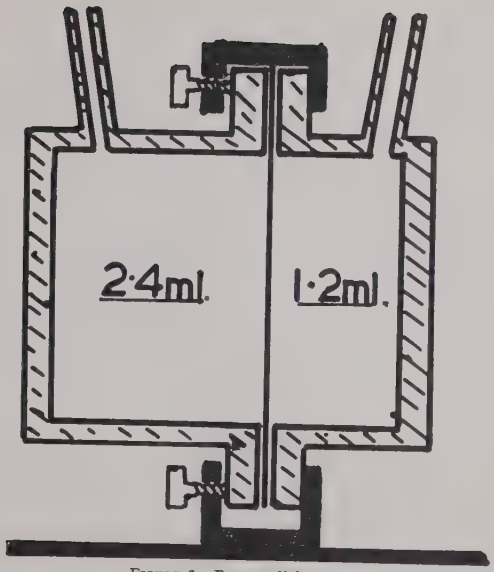

FIGURE 3. Perspex dialyzer.

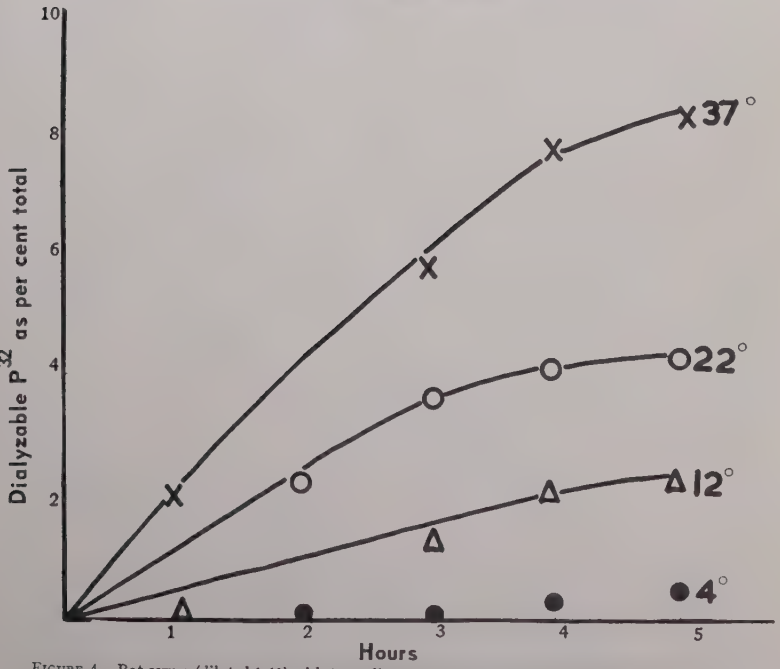

FIGURE 4. Rat serum (diluted 1:10) with 10 μg. lipopolysaccharide/ml. Effect of temperature.

40,000 g., consisting largely of lipopolysaccharide, was used in the experiments. This material had a specific activity varying from batch to batch between 5 to 20 μcurie./mg. This means that we could readily detect 1/1000 μg. by radioactive assay.

The method we used to test for the splitting of this lipopolysaccharide by serum was quite simple. The dilutions of serum and lipopolysaccharide (usually 10 μg./ml.) were placed in 1 compartment of small perspex dialyzers, separated by a cellophane membrane from the other compartment containing twice the volume of saline solution plus 0.1 per cent gelatine (FIGURE 3). The radioactive P^{32} dialyzing into the saline is a measure of the amount of lipopolysaccharide that has reacted.

It is evident that the splitting reaction has many of the characteristics of an enzyme reaction. For example, the splitting reaction is dependent on temperature (FIGURE 4).

It should be stressed at this point that the rate of accumulation of P^{32} in the saline solution was determined by the rate of dialysis and was not an indication of the rate of splitting except that this was faster than the rate of dialysis.

There was also a sharp pH optimum for the reaction (FIGURE 5). Heating at 56° C. for 20 minutes completely destroyed the ability of the serum to split

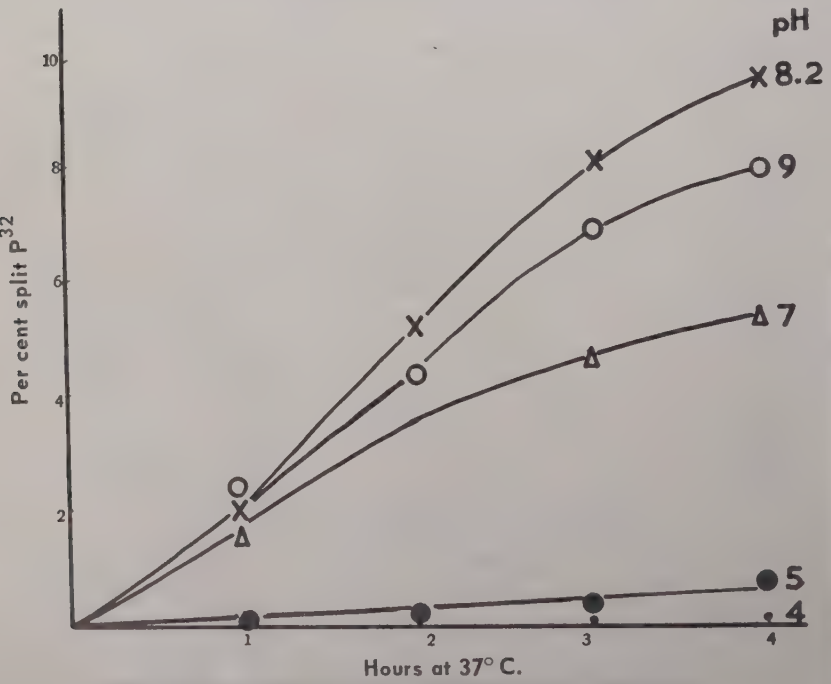

FIGURE 5. Rat serum (diluted 1:10) with 10 μg./lipopolysaccharide/ml. Effect of pH.

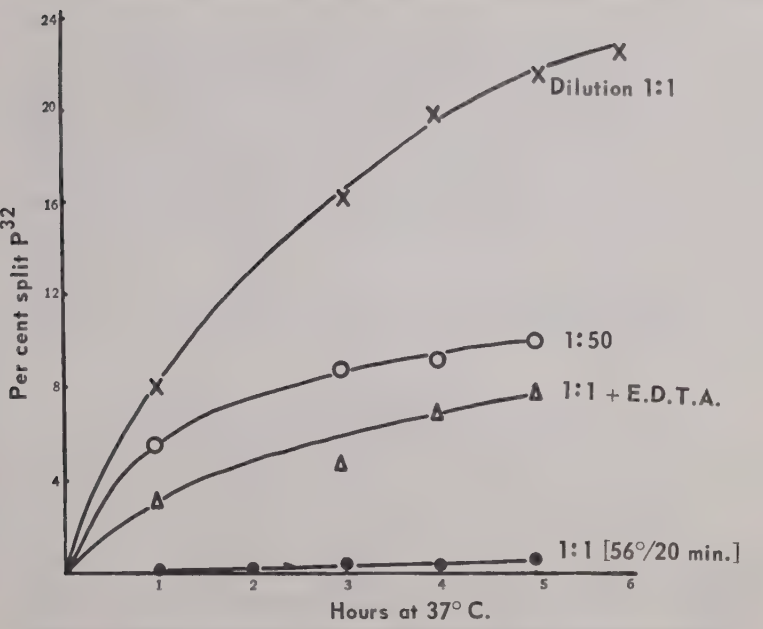

FIGURE 6. Liberation of dialzable P^{32} from lipopolysaccharide by saline dilutions of rat serum.

the lipopolysaccharide. This heat lability was not due to destruction of hemolytic complement, since mouse serum was very active in spite of its lack of whole hemolytic complement. The presence in I.R.C. 50 resin-treated serum of 0.0005 M ethylenediaminetetraäcetic acid (E.D.T.A.) to chelate divalent ions very greatly reduced the efficiency of the reaction (FIGURE 6).

Once the liberation of dialyzable P^{32} had ceased, the addition of fresh serum resulted in a further small release of phosphorus, indicating that the reaction came to a halt due to an inactivation of the enzyme and not due to a shortage of substrate.

All of the dialyzable P^{32} that was liberated was precipitated together with added phosphate when a precipitate of ammonium phosphomolybdate was formed *in situ*. It was presumed from this that the P^{32} was split off in the form of phosphate. This strongly suggested that the enzyme causing this cleavage is a phosphatase.

Is there any relationship between this enzyme and properdin or the alkaline phosphatase that can be measured in serum by the hydrolysis of phenol phosphate? All 3 of these activities declined following the injection into mice of rather large doses of bacterial lipopolysaccharide (TABLE 3).

In spite of this it was evident that at least 2 of these were distinct activities, since it is possible to have sera with great variations in King Armstrong alkaline phosphatase, all of which have the same efficiency in splitting lipopolysaccharide (TABLE 4). Further proof of this difference was provided by an experiment in

Table 3

The Properdin, Alkaline Phosphatase, and Lipopolysaccharide-Splitting Abilities of Mouse Sera Taken at Varying Times after the Injection of Lipopolysaccharide

Time after injection of lipopolysaccharide	Properdin (units/ml.)	Alkaline phosphatase (K.A.* units)	Per cent split P^{32} after 4 hrs. at 37° C. (serum diluted 1:20)
Normal controls	20–40	14	7.0
3 hours	20–40	11	6.0
6 hours	20–40	12	6.2
24 hours	10	9	3.0
48 hours	10	9	4.8

Twenty-four mice injected in vitro with 50 μg. crude lipopolysaccharide from *E. coli* 2380 and bled at intrevals in groups of 6. Serum in each group pooled.
* King Armstrong.

Table 4

Comparison Between the Average Levels of Properdin, Alkaline Phosphatase and Lipopolysaccharide-Splitting Activity of Different Sera

Serum (undiluted)	Per cent P^{32} split after 4 hrs.	Alkaline phosphatase (K.A. units)	Properdin (units)
Rat	18	50	50
Mouse	14	32	30
Human	14	17	8
Guinea pig	10	15	—
Rabbit	8	10	—
Human R_3	14	18	0
Case of hypophosphatemia	14	2	0
Case of biliary obstruction	14	60	0
Crude properdin	6–15	20–45	0–4

which the splitting of lipopolysaccharide by rat serum was followed in the presence of a hundredfold excess of phenol phosphate. The amount of split phosphate was just the same as in a control without phenol phosphate. It seemed likely also that properdin and lipopolysaccharide P^{32} splitting activity were separate, since human R_3 had the same activity as the serum from which it was derived. It is interesting that samples of crude properdin prepared from human or bovine sera by A. C. Wardlaw possessed quite high lipopolysaccharide splitting activity, as well as high values for King Armstrong phosphatase. It is evident that many substances other than properdin are adsorbed by and eluted from zymosan, and the mere demonstration that any serum activity is removed by treatment with zymosan is not proof that the activity is due to properdin. Much further work is required before we can decide what part, if any, this serum-phosphatase reaction plays in the destruction or detoxification of bacteria *in vivo*.

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THE EFFECT OF VARIOUS SUBSTANCES ON RESISTANCE TO EXPERIMENTAL INFECTIONS

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The parenteral administration of nonspecific agents for the stimulation of host resistance to bacterial infections has been investigated for many decades. The main efforts have been directed toward the enhancement of leukocytosis and phagocytosis, the induction of local inflammation, and the stimulation of an increase in production of natural or acquired antibodies or in the bactericidal power of serum. A wide variety of these nonspecific substances have been used clinically with varying success. These have been reviewed by Petersen[1] and by Perla and Marmorston,[2] and they include such diverse preparations as animal and plant proteins, protein split products, enzymes, bacterial extracts and vaccines, tissue and leukocytic extracts, and colloidal metals.

In 1923 Gay and Morrison[3] demonstrated that the introduction of a mild irritant such as meat-infusion broth into the pleural cavity protected rabbits against a lethal dose of *Streptococcus pyogenes* injected 24 hours later. The accumulation of clasmatocytes in the thoracic cavity apparently accounted for this increased resistance. In the same year Opie[4] reported that a peritonitis induced with aleuronat, a plant protein, protected animals against intraperitoneal infection with streptococci introduced 2 days later. These results were associated with an increase in macrophages in the peritoneal exudate. Menkin[5] has shown that potent chemical irritants such as aleuronat also stimulate the rapid formation of a fibrinous network, favoring development of a lymphatic blockade that contributes to the host defenses. In a more recent monograph, Menkin[6] has summarized his studies of chemical constituents in exudates that influence accumulation of phagocytes in inflamed areas and that increase capillary permeability. According to Nakahara,[7] mice were rendered refractory to intraperitoneal infection with *Diplococcus pneumoniae* and other pathogens if they received an injection of olive oil in the same site 2 days earlier. The oil produced an exudate containing about 75 per cent macrophages. More recently, Nungester and Ames[8] successfully controlled pneumococcal infections in mice with quaternary ammonium compounds that were devoid of bacteriostatic activity but were capable of stimulating phagocytosis *in vitro*. Konowalchuk and his associates[9] reported that intravenous administration of a preparation of colloidal sulfur 2 to 7 days before an intraperitoneal challenge with *D. pneumoniae* gave a high degree of protection but was without effect if the preparation was given simultaneously with the bacteria. Since this preparation was inhibitory to *D. pneumoniae* and other organisms *in vitro*, the protection was ascribed to a delayed antimicrobial action *in vivo*. The use of hog gastric mucin as an adjuvant for enhancing experimental infections, particularly in the abdominal cavity, is well known (Nungester, Wolf, and Jourdonais,[10] and Miller[11]). The literature on this subject has been reviewed by Olitzki.[12] When mucin is injected 24 to 48

hours prior to infection, however, mice are rendered more resistant to *Neisseria meningitidis* (Miller and Castles[13]). Mucin or peanut oil also protected animals against infection with *Salmonella typhosa* (Lambert, Smith, and Richley[14]). Hestrin and his associates[15] were able to enhance intraperitoneal infections with the same organism by administering native levans and dextrans by the intravenous route. An increased susceptibility of mice to *Salmonella typhimurium* and other pathogens following injection of certain inhibitors or intermediates of the tricarboxylic acid cycle has been studied by Berry and Mitchell.[16, 17] In a recent publication, Berry [18] established the fact that the bacteria multiplied more rapidly *in vivo* under these conditions and suggested that this growth acceleration might be attributable to an *in vivo* enhancement of the nutritive environment on which the bacteria were dependent, rather than on a lowering of the host cellular defenses. A yeast extract, malucidin, has been described by Parfentjev and his associates[19, 20] that, if administered a day or 2 in advance, renders mice resistant to infection with several gram-negative bacteria and to *Candida albicans*. The active principle is described as a protein fraction. Pillemer and his group[21, 22] have reported that zymosan, a polysaccharide prepared from yeast, renders animals more susceptible when injected a few hours before infection. A resistance to infection was observed, however, when the zymosan preceded the infection by 2 to 5 days. These investigators suggested that these alterations in the pattern of the resistance may be associated with the fall and rise in the titer of properdin, a recently described serum protein that interacts with zymosan *in vitro*. Rowley[23] observed similar changes in resistance to *Escherichia coli* infections in mice that had been pretreated with bacterial cell walls prepared from *E. coli* or *S. typhimurium*. These preparations, like zymosan, also were capable of depleting properdin from serum *in vitro*.

We have tested zymosan, malucidin, mucin, certain native dextrans and levans, polysaccharides from *D. pneumoniae* and gram-negative bacteria, and a preparation of colloidal sulfur, for their effect upon various experimental infections. All of these substances modified the course of infection provided the test conditions were optimal with regard to dosage, dosage time, and route of injection.

Experimental

Materials and methods. CF1* female mice weighing from 16 to 20 grams were used unless otherwise stated. The standard *Klebsiella pneumoniae* AD infection was done intraperitoneally with 0.5 ml. of a 10^{-5} brain-heart infusion broth dilution of a 6-hour brain-heart infusion broth culture from a stock culture. The stock culture was passed in mice once a week. Other infections will be described in connection with the experiment in which they were used, since they were not standardized.

In each experiment a difference in response between a treated and nontreated group of animals was measured. It was necessary to adopt criteria for judging whether this difference was significant. For this purpose we used

* Carworth Farms, New City, N. Y.

the tables of Mainland and Murray[24] to decide whether survival ratios were different, and the *t*-test was used for comparison of mean-survival times.

Zymosan

Zymosan is an insoluble carbohydrate complex prepared from fresh yeast cells by digestion with trypsin and extraction with water and alcohol. It inactivates selectively *in vitro* the third component of serum complement (C'3) in the presence of magnesium ions (Pillemer and Ecker[25] and Pillemer *et al.*[21]). The preparation used in our experiments, lot LE-1, was supplied by Merle Querry of the Biological Development Group of the Lederle Laboratories Division, American Cyanamid Company, Pearl River, N. Y. This preparation, in concentrations as high as 4 mg./ml., exhibited no *in vitro* antimicrobial activity against 10 species of gram-positive and gram-negative bacteria and 6 species of yeasts and filamentous fungi.

Zymosan was titrated in groups of mice intraperitoneally 48 hours before they were given the standard *K. pneumoniae* AD challenge. In this experiment, shown in TABLE 1, 6 mg. of zymosan killed 2 of 20 mice before they were infected. Significant protection was afforded by 1.5 mg. The median protective dose, with 19/20 confidence limits, was 1.88 (2.71 to 1.32) mg./mouse.

There were 2 possible approaches to the problem of the duration of the effect of the zymosan. One approach was to inject groups of mice with zymosan at various times, preferably on a logarithmic scale, and then to challenge all of the groups at the same time with the same suspension of *Klebsiella*. The other approach was to inject all of the mice with zymosan at the same time and then challenge groups of them at various intervals, together with suitable controls. The advantage of the second method is that the mice form a uniform population and, if a group of mice to be used as nontreated controls is drawn from the same supply at the same time as the mice treated with zymosan, then it is possible to compare the effect of the standard infection on the treated versus the nontreated groups at each time interval. This was done as shown

TABLE 1

EFFECT OF ZYMOSAN GIVEN INTRAPERITONEALLY 48 HOURS BEFORE AN INFECTION WITH *K. PNEUMONIAE* AD

Zymosan, mg.	Survival at 14 days	
	Alive/Total	Mean survival time, hours
6	16/18	124
3	15/20	78
1.5	6/20	75
0.75	3/20	43
0.38	0/20	30
0.19	2/20	29
0.095	1/20	39
Nontreated controls	0/20	31

Virulence titration:
One-half ml. of a 10^{-6} dilution 3/5
Infecting dose: 900 cells

Table 2
Duration of Protective Effect of Zymosan

Time before infection, days	Survival at 14 days					
	Zymosan-treated				Nontreated	
	Intraperitoneal		Intravenous			
	Alive/Total	Mean survival time, hour	Alive/Total	Mean survival time, hours	Alive/Total	Mean survival time, hours
2	19/20	64	2/20	39	0/20	24
4	13/20	142	1/20	48	2/20	32
8	4/20	123	3/20	36	1/20	27
16	1/20	37	5/20	35	0/20	36
32	4/20	62	7/20	54	3/20	37
Virulence titration: 10^{-6}				2 days	1/5	48
				4 days	5/5	—
				8 days	5/5	—
				16 days	5/5	—
				32 days	5/5	—

in TABLE 2. One hundred mice were treated with 3 mg. of zymosan intraperitoneally and another 100 were given 0.5 mg. of zymosan intravenously. A third group of 100 mice from the same lot were kept as controls. Twenty mice from each group were challenged with the standard *K. pneumoniae* AD culture at 2, 4, 8, 16, and 32 days after treatment. The intraperitoneally treated groups had significantly more survivors than the controls at 2 and 4 days after treatment and had a significantly prolonged survival time at 8 days, but beyond that they showed no difference from the controls. The intravenously treated groups showed no difference either in numbers of survivors or in survival time from the nontreated controls.

In order to learn how much variation could be expected from group to group within an experiment and between different experiments, 2 experiments were done in which 5 groups of 20 mice each were given 3 mg. of zymosan intraperitoneally, 5 groups were given 0.5 mg. intravenously, and 5 similar groups were used as nontreated controls. Forty-eight hours after treatment these mice were infected with the standard *K. pneumoniae* AD infection. The results of these 2 tests, given in TABLE 3, show considerable variation between groups within the treatments. The intraperitoneal treatment, however, resulted in about 60 per cent survival compared with 6 per cent for the nontreated controls and a threefold increase in mean survival time for the intraperitoneally treated group over the controls. The effect with the intravenously treated groups was very slight. In fact, if either experiment was considered alone, the number of survivors in the treated groups was not significantly greater than that in the controls. If, however, the results of the 2 experiments were pooled, the treated groups showed a difference that was significant at the 5 per cent level of probability.

Perhaps the most useful parenteral route of administration for drugs is the intramuscular. This route is technically very impractical in mice, but the

TABLE 3

EFFECT OF 3 MG. OF ZYMOSAN INTRAPERITONEALLY OR 0.5 MG. INTRAVENOUSLY GIVEN 48 HOURS BEFORE INFECTION WITH *K. PNEUMONIAE* AD

Route	Survival at 14 days			
	Experiment 1		Experiment 2	
	Alive/Total	Mean survival time, hours	Alive/Total	Mean survival time, hours
Intraperitoneal	15/20	83	10/20	122
Intraperitoneal	4/20	89	13/20	103
Intraperitoneal	18/20	136	13/20	140
Intraperitoneal	17/20	104	9/20	115
Intraperitoneal	13/20	65	19/20	112
Intravenous	4/20	50	1/20	39
Intravenous	6/20	52	1/20	33
Intravenous	4/20	56	0/20	46
Intravenous	4/20	59	3/20	45
Intravenous	3/20	46	2/20	51
None	3/20	46	0/20	32
None	0/20	45	0/20	27
None	3/20	40	0/20	31
None	1/20	43	0/20	24
None	4/20	45	2/20	23
Total 3 mg. intraperitoneal	67/100	95	64/100	118
Total 0.5 mg. intravenous	21/100	53	7/100	43
Total nontreated	11/100	44	2/100	27
Combined total:				
Three mg. intraperitoneal	131/200			
One-half mg. intravenous	28/200			
Nontreated	13/200			
Virulence titration:				
One-half ml. 10^{-6}	4/9		5/10	
One-half ml. 10^{-7}	5/5		5/5	
Infecting dose:	1700 cells		1000 cells	

subcutaneous route is easily done. We therefore tested zymosan for its effect by subcutaneous administration. A preliminary experiment showed that 9 mg. per mouse was well tolerated, so we set up an experiment in which this dose was administered subcutaneously at various times from one-half hour to 8 days before the standard *K. pneumoniae* AD infection. The results of this experiment are given in TABLE 4. Significant protection was evident when the zymosan was given at 1, 2, and 4 days, but not at one-half hour or 8 days before infection.

The protective effect of zymosan given intraperitoneally 1 to 8 days before infection with *K. pneumoniae* AD has been clearly shown, but the effect of 3 mg. of zymosan given intraperitoneally at 2 hours or less before infection was examined in a separate experiment (TABLE 5). In this experiment the challenge dose was 0.5 ml. of a 10^{-6} dilution of the standard *K. pneumoniae* AD culture. It was evident that the zymosan pretreatment actually depressed the resistance of the animals below that of the controls.

Since we had shown that pretreatment with 3 mg. of zymosan given intraperitoneally 48 hours before infection would increase resistance to infection, and that the same dose given shortly before infection would depress resistance,

TABLE 4

EFFECT OF 9 MG. OF ZYMOSAN GIVEN SUBCUTANEOUSLY AT VARIOUS TIMES BEFORE INFECTION WITH *K. PNEUMONIAE* AD

Number of days before infection	Survival at 14 days	
	Alive/Total	Mean survival time, hours
8	3/15	32
4	7/15	64
2	10/15	187
1	7/15	71
½ hour	0/15	35
Three mg. intraperitoneally 2 days	7/15	120
Nontreated controls 10^{-4}	0/15	32
Virulence titration:		
One-half ml. of a 10^{-5} dilution	0/10	
One-half ml. of a 10^{-6} dilution	4/5	

TABLE 5

EFFECT OF 3 MG. OF ZYMOSAN GIVEN INTRAPERITONEALLY AT SHORT INTERVALS BEFORE INFECTION WITH *K. PNEUMONIAE* AD

Time before infection, minutes	Survival at 14 days	
	Alive/Total	Mean survival time, hours
120	0/10	34
60	1/10	36
30	2/10	31
15	0/10	51
Nontreated controls 10^{-6}	10/10	—
Virulence titration:		
One-half ml. of a 10^{-4} dilution	0/10	
One-half ml. of a 10^{-7} dilution	9/10	

we believed it would be of interest to learn what effect a postinfection dose of zymosan would have on the resistance induced by an intraperitoneal dose of zymosan given 48 hours before infection.

This was done in the experiment described in TABLE 6. Five groups of mice were pretreated with 3 mg. of zymosan intraperitoneally 48 hours before a standard *K. pneumoniae* AD infection, and then, 4 hours after infection, 4 groups were given 3.0, 0.75, 0.18, or 0.045 mg. of zymosan intraperitoneally. Four similar groups of mice infected at the same time but not pretreated with zymosan were given corresponding doses of zymosan to learn whether post-infection treatment would confer protection. The results were clear-cut. A postinfection dose of 0.75 mg. of zymosan abolished the protective effect of the preinfection dose, although the treated mice had a significantly longer mean survival time than the nontreated. When the postinfection dose was reduced to 0.18 mg. both the number of survivors and the mean survival time were different from the controls. None of the groups that received only a postinfection treatment had any survivors and, in fact, the groups receiving the 2 larger doses had mean survival times significantly shorter than those of the nontreated controls.

TABLE 6

EFFECT OF A POSTINFECTION DOSE OF ZYMOSAN ON THE SURVIVAL OF MICE GIVEN ZYMOSAN INTRAPERITONEALLY 48 HOURS BEFORE INFECTION WITH *K. PNEUMONIAE* AD

Zymosan before infection, mg.	Zymosan after infection, mg.	Survival at 14 days	
		Alive/Total	Mean survival time, hours
3	—	10/14	66
3	3.0	1/15	64
3	0.75	1/15	97
3	0.18	6/14	86
3	0.045	7/15	90
—	3.0	0/15	19
—	0.75	0/15	18
—	0.18	0/15	23
—	0.045	0/15	30
Nontreated controls		1/15	33
Virulence titration:			
One-half ml. of a 10^{-6} dilution		0/5	
One-half ml. of a 10^{-7} dilution		4/5	
Infecting dose: 4200 cells			

In view of the fact that an intraperitoneal injection of zymosan would actually depress the resistance of the animal for at least 2 hours it was desirable to know whether such a depression of resistance could be counteracted by an antibiotic. To learn whether this could be done, 5 groups of mice were given a 3 mg. dose of zymosan 2 hours before infection with 0.5 ml. of a 10^{-6} dilution of the standard *K. pneumoniae* AD culture. Three groups were given a single intraperitoneal dose of tetracycline hydrochloride with the zymosan. This dose was 0.8, 0.4 or 0.2 mg. per mouse. Three similar groups of mice received only the tetracycline in corresponding dosage 2 hours before infection. The results of this experiment are given in TABLE 7. Here it is shown that the pretreatment with zymosan clearly depressed the resistance of the mice sufficiently so that 0.2 mg. of tetracycline was not able to save as many mice as survived in the nontreated controls, but 0.4 mg. of the antibiotic did counteract both the infection and the depression of resistance induced by pretreatment with zymosan.

In order to learn whether the resistance to infection, which was induced by pretreatment with zymosan, would be effective against gram-positive cocci, we gave 2 groups of mice a 3 mg. dose of zymosan intraperitoneally. One group was dosed 24 hours and the second group 48 hours before infection with 0.5 ml. of a 10^{-3} dilution of a culture of *Streptococcus pyogenes* C203. This culture was grown for 18 hours at 37°C. in brain-heart infusion broth and had not been recently passed through mice so that it was not highly virulent, as shown by the fact that there were 2 survivors in the nontreated controls and that 3 of 5 mice infected with 0.5 ml. of a 10^{-4} dilution survived. There was clearly an increased resistance to the infection in the 2 treated groups (TABLE 8), since 13 of 20 mice, pretreated at 48 hours, and 14 of 20 mice, pretreated at 24 hours, survived.

In additional experiments we were able to show that pretreatment with

Table 7

Effect of Tetracycline Treatment on Mice Given Zymosan Intraperitoneally 2 Hours Before Infection with *K. pneumoniae* AD

Zymosan, mg.	Tetracycline, mg.	Survival at 14 days	
		Alive/Total	Mean survival time, hours
3	None	0/20	25
3	0.8	20/20	—
3	0.4	20/20	—
3	0.2	6/20	57
None	0.8	19/20	88
None	0.4	19/20	88
None	0.2	18/20	76
Nontreated controls		9/20	96

Virulence titration:
One-half ml. of a 10^{-6} dilution 1/20
One-half ml. of a 10^{-7} dilution 4/5
Infecting dose: 800 cells

Table 8

Effect of 3 mg. of Zymosan Given Intraperitoneally Before Infection with *S. pyogenes* C-203

Time before infection, hours	Survival at 14 days	
	Alive/Total	Mean survival time, hours
48	13/20	63
24	14/20	51
Nontreated controls	2/20	30

Virulence titration:
One-half ml. of a 10^{-4} dilution 3/5
One-half ml. of a 10^{-5} dilution 3/5
One-half ml. of a 10^{-6} di ution 5/5

zymosan would increase the resistance of mice to infection with *Proteus vulgaris* OX-19, *Pseudomonas fluorescens* Ps2097, and *Micrococcus pyogenes* var. *aureus* NY-104, as shown by a significant difference in the numbers of survivors of treated over nontreated mice. We were also able to show an increase in mean survival time of treated over nontreated mice against *Bacillus anthracis* 20S. We did not show any effect on an infection with *Diplococcus pneumoniae* IR type 1 or *Candida albicans* in mice, but we did show protection against *Pasteurella multocida* P-652 in chicks.

Malucidin

The malucidin supplied to us by I. A. Parfentjev was a gray slurry containing about 40 mg. of solids per ml. It did not take a methylene blue stain, and microscopic examination of a wet mount revealed mainly an amorphous material, although an occasional yeast cell could be seen.

An experiment was done in which malucidin was tested for its ability to enhance resistance to *Brucella abortus* 2308, a fully virulent human strain. The mice were infected intraperitoneally with 0.2 ml. of a suspension contain-

TABLE 9

EFFECT OF MALUCIDIN BY VARIOUS ROUTES ON AN INFECTION WITH *B. ABORTUS* 2308

Malucidin, mg. × 3 doses	Survival at 21 days		
	Route	Alive/Total	Mean survival time, days
4	Intraperitoneal	5/5	—
1	Intraperitoneal	4/5	3.0
0.4	Intraperitoneal	8/10	4.0
4	Subcutaneous	1/10	2.8
1	Subcutaneous	0/10	2.0
0.4	Subcutaneous	1/10	2.5
1	Intravenous	1/8	1.9
0.4	Intravenous	1/10	2.1
Nontreated controls		0/10	3.3
Virulence titration:			
Two-tenths ml. of a 1:2 dilution		2/5	8.3
Two-tenths ml. of a 1:10 dilution		2/5	9.0

ing 40 × 10^8 viable organisms per ml. This suspension was prepared by harvesting the 4-day growth from tryptose agar slants in 10 ml. of peptone water. Groups of mice were treated with 4, 1, or 0.4 mg. of malucidin by the intraperitoneal, subcutaneous, or intravenous route. The volumes used were 0.25 ml. for the intraperitoneal and subcutaneous, and 0.2 ml. for the intravenous. Three doses of the indicated amount were given, one 24 hours before, and one at 48 hours and one at 96 hours after infection. At the end of 21 days the survivors were killed with chloroform, and the spleens were removed aseptically, hemisected, and streaked on tryptose agar plates containing 1:700,000 crystal violet. The entire spleen was then weighed. The plates were incubated for 6 days at 37° C. under 10 per cent carbon dioxide before being examined for colonies of *Brucella*.

In terms of survival, even the smallest dose of malucidin given intraperitoneally was highly effective, but all of the survivors continued to harbor *Brucella* in their spleens. By the subcutaneous or intravenous routes the malucidin, in the doses tested, was ineffective (TABLE 9).

In other experiments we were able to show that malucidin given intraperitoneally at 24 or 48 hours before infection with *Proteus vulgaris* OX-19, *Proteus mirabilis* HD, or *Streptococcus pyogenes* C-203 gave significant protection.

Mucin

We compared the effect of varying doses of mucin (Wilson 1701W), given intraperitoneally 48 hours before the standard *K. pneumoniae* AD infection, with the standard 3 mg. intraperitoneal dose of zymosan. These results, shown in TABLE 10, indicate that a dose of mucin eightfold larger than that of zymosan confers considerably less protection than the zymosan, although smaller doses did give some prolongation of mean-survival time.

We then repeated the above experiment using *S. pyogenes* C-203 as the challenge organism. Here the results were very different. Even 0.75 mg. of mucin gave results that were not significantly lower than those for 3 mg. of

TABLE 10

EFFECT OF VARYING DOSES OF MUCIN GIVEN INTRAPERITONEALLY 48 HOURS BEFORE INFECTION WITH *K. PNEUMONIAE* AD

Mucin, mg.	Survival at 14 days	
	Alive/Total	Mean survival time, hours
24	7/20	52
12	1/20	41
6	0/20	35
3	0/20	43
1.5	2/20	41
0.75	1/20	40
Zymosan 3 mg.	15/19	138
Nontreated controls	0/20	33

Virulence titration:
One-half ml. of a 10^{-6} dilution 2/5
One-half ml. of a 10^{-7} dilution 4/5
Infecting dose: 215 cells

TABLE 11

EFFECT OF VARYING DOSES OF MUCIN GIVEN INTRAPERITONEALLY 48 HOURS BEFORE INFECTION WITH *S. PYOGENES* C-203

Mucin, mg.	Survival at 14 days	
	Alive/Total	Mean survival time, hours
24	18/20	148
12	18/20	104
6	15/20	88
3	17/20	48
1.5	15/20	40
0.75	12/20	56
Zymosan 3 mg.	16/20	60
Nontreated controls	2/20	41

Virulence titration:
One-half ml. of a 10^{-7} dilution 4/5
One-half ml. of a 10^{-8} dilution 3/5
Infecting dose: 145 cells

zymosan. It will be noted that this was a fully virulent culture of *S. pyogenes* C-203, since the challenge dose of 0.5 ml. of a 10^{-6} dilution killed 18 of 20 nontreated mice (TABLE 11).

Dextrans and Levans

H. M. Tsuchiya, of the Agricultural Research Service Laboratory at Peoria, Ill., supplied us with several samples of dextran and levan. We dosed groups of mice intravenously or intraperitoneally 48 or 6 hours before a standard *K. pneumoniae* AD infection. The preparations used differed widely in their ability to increase resistance, as will be seen in TABLE 12. Dextran B-742 given in a 50-mg. dose intraperitoneally 48 or 6 hours before infection gave good protection. A 25-mg. dose of the same substance given intravenously 6 hours before infection also gave good protection. If given 48 hours before infection, however, dextran B-742 was much less effective, although the mean-

TABLE 12

EFFECT OF DEXTRANS AND A LEVAN ON AN INFECTION WITH *K. PNEUMONIAE* AD

Preparation	Dose, mg.	Route	Time before infection, hours	Survival at 14 days	
				Alive/Total	Mean survival time, hours
Dextran B-742	50	Intraperitoneal	48	13/20	105
	50	Intraperitoneal	6	17/20	104
	25	Intravenous	48	3/20	62
	25	Intravenous	6	11/20	90
Dextran B-1146	12.5	Intraperitoneal	48	2/20	49
	12.5	Intraperitoneal	6	14/20	59
	6.25	Intravenous	48	1/20	40
	6.25	Intravenous	6	1/20	38
Levan from B-512, fraction A	25	Intraperitoneal	48	0/20	41
	25	Intraperitoneal	6	5/20	65
	12.5	Intravenous	48	0/16	39
	12.5	Intravenous	6	0/20	46
Zymosan	3	Intraperitoneal	48	15/20	137
Nontreated controls		—	—	2/20	27

Virulence titration:
One-half ml. of a 10^{-6} dilution 1/5
One-half ml. of a 10^{-7} dilution 5/5
Infecting dose: 1000 cells

survival time was significantly prolonged. Dextran B-1146 formed a much more viscous suspension, so that the dose used was only one fourth of that used for B-742. This preparation gave good protection when 12.5 mg. were injected 6 hours before infection, but it prolonged survival time, significant at the 1 per cent level, only if the dose was given 48 hours before infection. An intravenous dose of 6.25 mg. either 48 or 6 hours before infection was without effect. Fraction A from a levan from B-512, given at 25 mg. intraperitoneally or 12.5 mg. intravenously at 6 or 48 hours before infection gave no significant difference in number of survivors, but it did increase the mean-survival time of the group dosed intraperitoneally 6 hours before infection by a significant amount.

Pneumococcus Polysaccharides

Some pneumococcus polysaccharides had also been reported by Pillemer *et al.*[22] to have absorbed properdin from serum, so they, too, were tried for their ability to increase resistance to infection. Since, like the dextrans, they form viscous suspensions, and since the supply was not large, we used a 5-mg. dose given intraperitoneally at 6 or 24 hours before a standard *K. pneumoniae* AD infection. Polysaccharides from types 12, 18, and 19, supplied to us by the Biological Production Section of the Lederle Laboratories Division, were used and, as can be seen in TABLE 13, the groups injected 6 hours before infection all had a significantly greater number of survivors than the nontreated controls. The groups injected at 24 hours before infection all had a significantly increased mean-survival time, but only the group injected with the type-18 polysaccha-

TABLE 13

EFFECT OF 5 MG. OF POLYSACCHARIDES FROM TYPE 12, 18, OR 19 PNEUMOCOCCUS GIVEN INTRAPERITONEALLY ON AN INFECTION WITH *K. PNEUMONIAE* AD

Pneumococcus type	Time before infection, hours	Survival at 14 days	
		Alive/Total	Mean survival time, hours
12	24	3/20	43
	6	7/20	44
18	24	6/20	57
	6	5/20	53
19	24	1/20	55
	6	15/20	54
Zymosan 3 mg.	48	16/20	60
Nontreated controls	—	0/20	32
Virulence titration: One-half ml. of a 10^{-6} dilution Infecting dose: 400 cells		5/5	

ride had a significant number of survivors. A polysaccharide from a type-14 pneumococcus could also be shown to increase resistance to a *Klebsiella* infection.

Polysaccharides from Gram-Negative Bacteria

It was reported by Pillemer and his associates[22] that polysaccharides from gram-negative bacteria would absorb properdin from serum. Also, Rowley[23] showed that a lipopolysaccharide from *E. coli* would enhance resistance to a virulent strain of *E. coli*. He was kind enough to supply us with a small amount of one of these lipopolysaccharides, and we were able to show that it would also increase resistance to our standard *K. pneumoniae* AD infection.

In this experiment, shown in TABLE 14, we injected groups of mice intravenously with 0.02 mg. or intraperitoneally with 0.09 mg. of the lipopolysaccharide at various times from 2 to 72 hours before the standard *K. pneumoniae* AD infection. The intravenous dose was chosen at the suggestion of Rowley, and the intraperitoneal dose was near the maximum tolerated dose, as shown by the death of 3 of the mice before infection.

The group of mice injected intravenously 24 hours before infection had a significantly greater number of survivors than the nontreated mice. None of the other groups had significantly more survivors than the controls, but the groups injected intravenously at 6 and 48 hours and those injected intraperitoneally at 24, 48, and 72 hours had a significantly prolonged mean-survival time.

A polysaccharide from a *Pseudomonas* species has been described by Nesset *et al.*[26] This material, known as Piromen,* was tested for its ability to increase resistance to the standard *K. pneumoniae* AD infection and also to see if it might, under the proper conditions, depress resistance to the infection. The doses used were 0.1 mg. intraperitoneally or 0.06 mg. intravenously.

* Kindly supplied to us by Myron Usdin of Baxter Laboratories, Morton Grove, Ill.

TABLE 14

EFFECT OF A LIPOPOLYSACCHARIDE FROM *E. COLI* ON AN INFECTION WITH *K. PNEUMONIAE* AD

Route and dose, mg.	Time before infection, hours	Survival at 14 days	
		Alive/Total	Mean survival time, hours
Intravenous	72	1/20	33
0.02	48	1/19	45
	24	8/20	75
	6	3/20	51
	2	0/20	31
Intraperitoneal	72	2/19	41
0.09	48	3/18	57
	24	3/20	58
	6	2/20	32
	2	0/20	20
Zymosan 3 mg. intraperitoneal	48	14/20	134
Nontreated controls	—	0/20	28
One-half ml. of a 10^{-6} dilution		1/5	60
One-half ml. of a 10^{-7} dilution		2/5	83

Infecting dose: 280 cells

TABLE 15

EFFECT OF A POLYSACCHARIDE FROM A *PSEUDOMONAS* SP. ON AN INFECTION WITH *K. PNEUMONIAE* AD

Route and dose, mg.	Time before infection, hours	Survival at 14 days	
		Alive/Total	Mean survival time, hours
Intraperitoneal	48	7/40	56
0.1	24	27/40	73
	6	3/20	51
Intravenous	24	2/40	47
0.06	6	14/20	53
Zymosan 3 mg. intraperitoneal	48	22/40	100
Nontreated controls	—	1/40	31
Virulence titration:			
One-half ml. of a 10^{-6} dilution		25/40	79

Infecting dose: 400 cells

In the first experiment the mice were treated 6, 24, or 48 hours before infection, as indicated in TABLE 15. The groups treated intraperitoneally at 24 hours or intravenously at 6 hours both had significantly more survivors than the non-treated controls. All of the treated groups had a significantly prolonged mean-survival time.

Three groups of mice received 0.1 mg. of the polysaccharide intraperitoneally, 0.06 mg. intravenously, or 3 mg. of zymosan intraperitoneally 2 hours before being infected with 0.5 ml. of a 10^{-6} dilution of the standard *K. pneumoniae* AD culture. The results, given in TABLE 16, show that the intraperitoneal dose of the polysaccharide and the zymosan depressed resistance to the infection, but that the intravenous dose did not.

TABLE 16

EFFECT OF A POLYSACCHARIDE FROM A *PSEUDOMONAS* SP. GIVEN 2 HOURS BEFORE INFECTION WITH *K. PNEUMONIAE* AD

Route and dose, mg.	Survival at 14 days	
	Alive/Total	Mean survival time, hours
Intraperitoneal, 0.1	6/40	60
Intravenous, 0.06	22/40	72
Zymosan, 3.0	0/40	28
Nontreated controls 10^{-6}	25/40	79
Virulence titration: One-half ml. 10^{-7} dilution Infecting dose: 40 cells	5/5	

Colloidal Sulfur

Konowalchuk, Hinton, and Reed[9a] published a series of papers in which they described a reaction product of cysteine and iron. This material was identified as colloidal sulfur, although there was some minor difference in the X-ray diffraction pattern. Ordinary colloidal sulfur was biologically inactive in their hands.

All of the materials that we had found to have a zymosanlike activity were very complex organic substances, so it was decided to prepare some material by the method described by Konowalchuk *et al.*[9] and test it for zymosanlike activity.

Ten gm. of cysteine hydrochloride and 2 gm. of ferric ammonium citrate were added to 20-liter lots of distilled water, and 5 N sodium hydroxide was used to bring the pH to 6.8. The solutions were autoclaved at 120° C. for 1 hour, allowed to cool overnight, and reautoclaved at 120°C. for 1 hour. They were then allowed to cool for several hours before being placed in the chill-room at about 4°C., where they were held for 5 days. A grey-white precipitate formed, leaving a clear supernate. The supernate was siphoned off, and the precipitate was centrifuged about 15 minutes at about 3000 rpm., washed several times with distilled water, and air-dried. The yield was about 250 mg. per 20 l., or about 12.5 per cent of the sulfur in the cysteine.

Two batches of material were tested, 1 intravenously and 1 intraperitoneally, since the amount of material per batch was small. Control groups were run at what we believed were critical times to see if the batches had the same amount of activity. A preliminary check indicated that 3 mg. of material would be well tolerated by the intraperitoneal route, while 1 mg. was near the maximum tolerated dose by the intravenous route. Accordingly, these doses were given at various times before infection. The time intervals chosen were those used by Hinton, Konowalchuk, and Reed[9c] except that, because of lack of material, we could not use all of the time intervals tested by these investigators.

TABLE 17 shows the result of this test against the standard *K. pneumoniae* AD infection. The 3-mg. intraperitoneal dose protected at 24, 54, and 78

TABLE 17

EFFECT OF "COLLOIDAL SULFUR" ON AN INFECTION WITH *K. PNEUMONIAE* AD

Colloidal sulfur batch No.	Route and dose, mg.	Time before infection, hours	Survival at 14 days	
			Alive/Total	Mean survival time, hours
A	Intravenous 1.0	120	6/14	75
A	Intravenous 1.0	96	8/14	72
A	Intravenous 1.0	78	4/15	74
B	Intravenous 1.0	78	5/15	80
A	Intravenous 1.0	54	7/14	67
A	Intravenous 1.0	24	4/13	54
A	Intravenous 1.0	4	7/14	42
B	Intraperitoneal 3.0	78	12/15	128
A	Intraperitoneal 3.0	78	14/15	160
B	Intraperitoneal 3.0	54	12/15	64
B	Intraperitoneal 3.0	24	12/15	40
B	Intraperitoneal 3.0	4	1/15	45
Zymosan	Intravenous 1.0	54	2/13	51
Zymosan	Intraperitoneal 3.0	54	8/15	82
Nontreated controls		—	2/15	46
Virulence titration:				
One-half ml. of a 10^{-6} dilution			0/5	62
One-half ml. of a 10^{-7} dilution			5/5	
Infecting dose: 1000 cells				

hours. The intravenous dose was less effective but, if all the groups were considered, it would seem that both preparations were active.

DISCUSSION

In this investigation we have shown that 6 complex organic substances, 5 of them of microbial origin and the sixth from hog stomach, and 1 inorganic substance, colloidal sulfur, can protect mice from an otherwise lethal bacterial infection. All of the substances will protect by the intraperitoneal route against an intraperitoneal infection. An enhancement of resistance, however, was also shown with zymosan injected subcutaneously. Dextrans, polysaccharides from gram-negative bacteria, and colloidal sulfur gave protection when administered by the intravenous route, although zymosan gave very poor protection and malucidin gave no protection by this route. We have also shown, in experiments not reported in detail here, that zymosan given intraperitoneally will protect against an infection given by the intravenous route. It would seem unlikely, therefore, that the enhancement of resistance is due to phenomena occurring entirely within the peritoneal cavity, although preliminary work has shown an initial depression and then a greatly increased number of leukocytes in the peritoneal cavity after the injection of zymosan.

Several of the substances used by us have been demonstrated by Pillemer *et al.*[22] to absorb properdin from serum, but we have not determined what, if any, relation may exist between the enhancement of resistance and the properdin system. Such a relationship might be shown, either directly by injection of properdin, or indirectly by the demonstration of a high properdin

titer in mice from groups that showed high resistance to infection. Our attempts to show a relationship by either method were unsatisfactory.

Zymosan and a polysaccharide from a *Pseudomonas* species were shown to depress resistance to infection when given shortly before the infection, and zymosan depressed resistance when given 4 hours after the infection.

No quantitative dose-effect relationship was determined among the dextrans or the pneumococcus polysaccharides, nor did we try to compare the effects of the various substances quantitatively.

Summary

We have shown that zymosan, malucidin, mucin, some native dextrans, polysaccharides from types 12, 14, 18, and 19 pneumococci, polysaccharides from *E. coli* and a *Pseudomonas* sp. and a preparation of colloidal sulfur, when given by a route and at a time before infection that differs from substance to substance, increase resistance to infection with *K. pneumoniae* AD. Zymosan, malucidin, and mucin were also shown to increase resistance to *S. pyogenes*, and zymosan and malucidin were shown to increase resistance to a number of other bacterial infections, both gram-positive and gram-negative.

Zymosan and a polysaccharide from a strain of *Pseudomonas* were shown to depress resistance to infection with *K. pneumoniae* when given shortly before the infection. In the case of zymosan, however, this depression of resistance could be counteracted by a dose of tetracycline.

A postinfection dose of zymosan was shown to abolish the increased resistance to *K. pneumoniae* induced by a preinfection dose of zymosan.

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THE EFFECT OF PERIPHERAL VASCULAR COLLAPSE ON THE ANTIBACTERIAL DEFENSE MECHANISMS*

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Previous work has demonstrated that animals in experimental hemorrhagic shock produced by the elevated reservoir technique will regularly survive if the blood-volume deficit is restored after 2 hours at a fixed low level of blood pressure, and that animals will die with nearly equal regularity if the blood-volume deficit is not restored until after 4 to 5 hours at the same fixed low level of blood pressure.[1] The explanation for the difference between survival and recovery as a function of time, in our view, lies in the extent of the injury done to the bactericidal mechanisms by the deficient blood flow. As soon as shock develops, bacteria in the tissues, or normally and continuously invading from the respiratory or gastrointestinal tract, begin to produce toxins. These cannot be excreted during shock. As the shock continues, the antibacterial mechanisms, including those that destroy bacterial toxins, continue to deteriorate, so that the host becomes increasingly vulnerable to bacterial activity. Eventually the toxins accumulate in sufficient amount to render the circulation refractory to transfusion. Even if the response to transfusion is satisfactory, the host remains excessively vulnerable during the recovery period and does not acquire its normal antibacterial potential for about 48 hours. This paper summarizes the evidence obtained to date in support of this hypothesis.

Broad-range antibiotics given prior to inducing shock increased the survival rate of animals, transfused after exposure to hemorrhagic shock for 4 or more hours, from less than 20 per cent to 65 per cent or higher.[2] This was interpreted to mean that the development of the refractory state of the circulation was prevented because the generation of bacterial toxins was suppressed.

A more direct relationship between the refractory state and bacterial toxins was demonstrated in experiments in which a fatal peritonitis was produced in dogs by injecting a fecal suspension intraperitoneally.[3] By this technique a very severe form of hypovolemic septic shock is produced. The plasma-volume deficit is severe, and the cardiac output falls rapidly to very low levels, often well in advance of the fall in blood pressure, and death occurs in 3 to 9 hours. Therapy with plasma in sufficient volume to prevent a critical fall in plasma volume fails to secure any significant benefit. If a broad-range antibiotic is given before shock is induced, however, recovery occurs even though the shock is as prolonged and severe as in the unprotected animal.† Since

* This paper is a summary of investigations published, in press, or to be published by the following collaborators: J. Fine, E. D. Frank, H. A. Frank, E. Friedman, E. Gordon, H. Korman, S. Jacob, A. Rutenburg, S. Rutenburg, F. Schweinburg, and H. Weizel.

Expenses for the work described in this paper were provided in part by a contract with the Office of the Surgeon General, United States Army, through the Subcommittee on Shock, National Research Council, Washington, D. C., and in part by a grant from the National Institutes of Health, Public Health Service, Department of Health, Education, and Welfare, Bethesda, Md.

† The one exception to this is that the blood pressure does not fall to a very low level. The blood pressure tends to remain elevated in the untreated animal in burn shock[4] and tourniquet shock,[5] as well as in a profusely exudative septic type of shock, until the circulation begins to fail and prelethal collapse is imminent. This is probably due to the hemodynamic effects of the very high hematocrit, which is characteristic of these types of shock because the blood-volume deficiency arises from a loss of plasma without a significant loss of red cells.

the antibiotic served only to exclude bacterial toxins, and no other therapy was given, it follows that, in the absence of bacterial toxins, prolonged hypovolemic shock can be tolerated and still remain responsive to fluid volume therapy, in this case by the resorption of fluid from the peritoneal cavity.

Search for the offending bacteria in the living tissues or blood in the rat,[6] rabbit,[7] and dog[8] has failed. In the dog, clostridia are present in many tissues but, because neomycin was among the effective antibiotics tested, they cannot be the primary offenders. Nevertheless, bacteria are present and responsible for the development of the refractory state of the circulation as the following experiments indicate:[8]

(1) The livers were excised, with aseptic precautions, from normal dogs and from dogs dying of hemorrhagic shock refractory to transfusion. Mash prepared from "normal" liver or from "shocked" liver was injected into the peritoneal cavity of normal dogs. These dogs were found to tolerate the intraperitoneal injection of "normal" and "shocked" liver mash equally well, as indicated by the survival of 70 per cent of them. But dogs transfused after 2 hours of hemorrhagic shock did not respond to such injections as do normal dogs. They tolerated "normal," but not "shocked" liver. The "shocked" liver therefore contained a factor, not present in "normal" liver, that was lethal for a reversibly shocked dog that otherwise would have recovered. This factor was shown to be of bacterial origin by the further observation that the reversibly shocked dog was fully protected if given antibiotic. If the recipient in reversible shock is vulnerable to "shocked" liver, how much more vulnerable must the donor in more prolonged shock have been to this bacterial factor in his own tissues?*

With the foregoing observations as a background, we proceeded to investigate the status of the various components of the antibacterial defenses in hemorrhagic shock. The first defense tested was the ability to clear and destroy a challenge dose of bacteria.[8] Normal dogs and dogs transfused after 2 hours in hemorrhagic shock received intravenous doses (5 to 50 million cells) of a 24-hour culture of 1 of 3 species of bacteria. They were found to clear such bacteria with virtually equal facility, so that less than 10 bacteria per ml. of blood were recovered 6 hours after injection. Irreversibly shocked dogs did not clear the blood as well for, in about one half of them, the bacteria were rapidly multiplying by the sixth hour. The bactericidal powers of the irreversibly shocked dogs had therefore become much weaker than those of the reversibly shocked dogs because of the longer exposure to shock. More striking, however, was the difference between the bactericidal powers of the normal and the reversibly shocked dogs, for the normal dogs showed sterile blood the next day, whereas all the 2-hour-shock dogs showed bacteremia. The normal dogs recovered, but all the 2-hour-shock dogs died with bacteremia in 1 to 4 days. The 2-hour-shock dogs also died when the bacteria were given after transfusion at any time up to 24 hours. If given bacteria during the second 24 hours after transfusion, the 2-hour-shock dogs did not kill the bacteria as rapidly as did normal dogs, but they survived.

* The response to muscle mash was the same as to liver mash.

(2) The other defense mechanism studied was the phagocytic activity of granulocytes in plasma.[9] The phagocytic index of granulocytes taken from normal dogs and immersed in plasma from normal dogs served as a standard. Some of these normal dogs were then exposed to irreversible shock and others were transfused after 2 hours of shock. Successive samples of plasma from each dog in both groups during shock, and from the latter group during the recovery phase, were then tested for their effect on the phagocytic activity of normal granulocytes. A decline in the phagocytic index was observed in both groups. In the irreversibly shocked dogs the index continued to fall more and more as the shock continued. In the reversibly shocked dogs the decline was halted by the transfusion, and the next day the phagocytic index was back to or near the preshock value. This reaction was taken to signify a loss in the titer of a phagocytosis-promoting factor in plasma but, in view of recent data to be presented below, the loss of phagocytic activity is more likely to be caused by a toxin that appears in plasma in consequence of the shock state.

Further studies of the effect of plasma from shocked animals on the function of phagocytes were made as follows:[10] granulocytes were obtained from the peritoneal cavity of a rabbit 6 hours after the injection of beef-infusion broth. These granulocytes were tested for their capacity to ingest and destroy bacteria when immersed in plasma from normal and shocked rabbits. The plasma from the shocked rabbits was taken 4 hours after transfusion for hemorrhagic shock of 2 hours' duration. Granulocytes in normal plasma displayed a much higher phagocytic and lytic capacity than the same cells immersed in plasma from the shocked rabbit. Moreover, these cells showed gross morphologic injury only when immersed in the plasma from the shocked rabbit. This injury consisted of a gross malformation of the nucleus, which changed from a multilobular to a ringlike form with loss of nuclear detail, together with blurring and disruption of the cytoplasm (FIGURE 1). Cells so damaged, if washed 3 times in iced gelatin and Locke's solution and then immersed in normal plasma, remained injured morphologically and functionally. These data demonstrate that reversible hemorrhagic shock results in the development of a substance that is toxic to granulocytes. Macrophages are likewise injured morphologically by immersion in shock serum, but the degree of functional damage, if any, has not yet been determined.

Whether this leukotoxin injures the phagocytes of the animal from which the serum was obtained is not known, but indirect evidence that this is true is twofold:

(1) Macrophages harvested at 6, 12, 24, and 48 hours from the peritoneal cavity of normal rabbits irritated by the injection of beef-infusion broth were homogenized and their enzyme content determined quantitatively by appropriate colorimetric methods.[11] No change was observed in the amount of lipase, esterase, or alkaline phosphatase with these differences in time. The amount of β-glucuronidase and acid phosphatase increased steadily, however, up to 4 times the control value at the end of 48 hours. Corresponding data from rabbits transfused after 2 hours in hemorrhagic shock showed no increase in

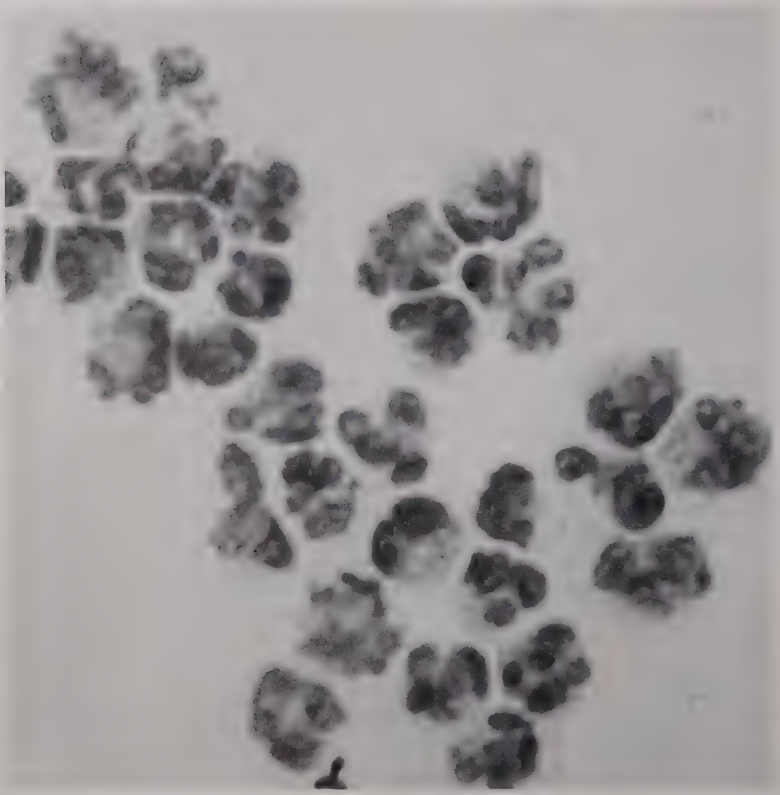

FIGURE 1. Granulocytes from the peritoneal cavity of the normal rabbit washed in iced gelatin and Locke's solution and then suspended in fresh plasma from a normal rabbit.

the content of β-glucuronidase or acid phosphatase at any time during the 48-hour period.

(2) Intravenous endotoxin rapidly induces leukopenia, which persists until death if the dose of endotoxin is lethal. Within 5 to 10 minutes after injecting the endotoxin, the granulocyte count falls to below 20 per cent of the total count and remains there or continues to fall still further until death. The lymphocytes constitute the remainder of the total count, and they show an absolute as well as a relative increase at first but, as the total count continues to fall, the total lymphocyte count may decline below the control value. The same effect is observed when the dose of endotoxin is sublethal, except that the leukopenia is not as severe and, after 4 hours or more, there is a beginning return to the normal total and differential count, which may be reached quickly or after some hours.

The behavior of the leukocytes in the blood of the rabbit exposed to hemorrhagic shock is almost the same as when endotoxin is given.[12] The leukopenia is not as marked and the fall in the granulocyte count not quite as rapid or steep. In the reversibly shocked rabbits the total and granulocyte counts do not recover until 18 to 24 hours after transfusion.

Whether or not the granulocytopenia represents destruction or immobilization in the peripheral circulatory bed, there is no doubt, as Miles and Niven have already shown,[13] that the shocked animal cannot deliver granulocytes to a site of challenge by an irritant during shock or, as we have noted, for some time after recovery of a normal hemodynamic status. We have attempted to quantitate this phenomenon by comparing the yield of cells from the peritoneal cavity of a normal rabbit 6 hours after injecting beef-infusion broth with the yield from a rabbit similarly injected immediately after transfusion for 2 hours of hemorrhagic shock.[14] The yield from the unchallenged peritoneal cavity of normal and shocked rabbits is 3 to 4 million cells, of which 65 to 70

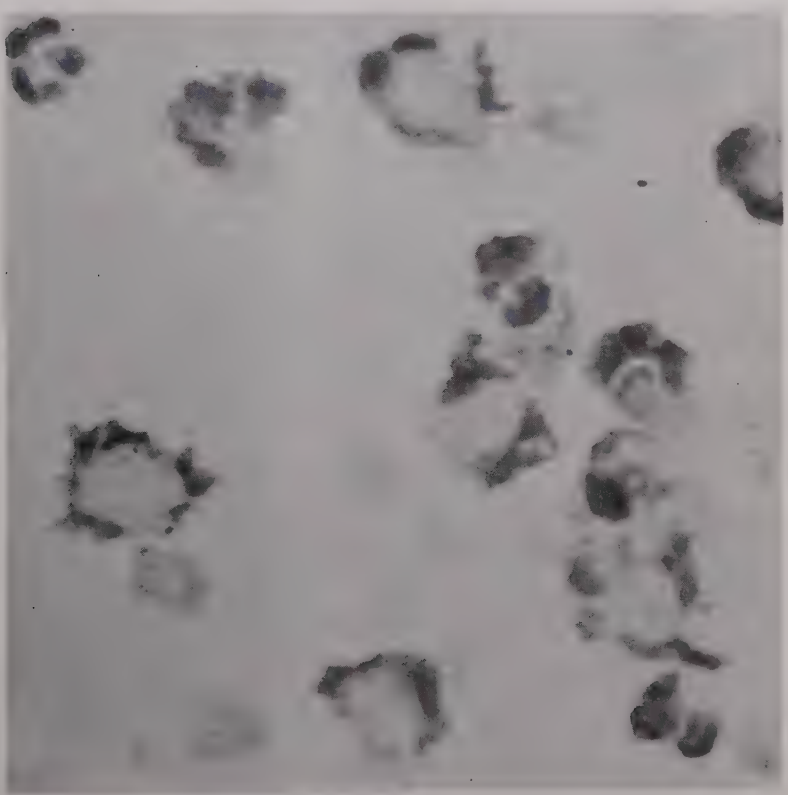

FIGURE 2. Granulocytes as in FIGURE 1 except that the plasma is from a rabbit 4 hours after transfusion for hemorrhagic shock of 2 hours' duration.

per cent are macrophages, 20 per cent are granulocytes, and 10 per cent are lymphocytes. An average of 100 million cells is obtained from the challenged normal rabbit. Of these, 93 per cent are granulocytes and 7 per cent are macrophages. In the challenged shocked rabbit, however, the total number of cells that can be washed out of the peritoneal cavity is no greater than can be washed out of the unchallenged peritoneal cavity, nor is there any change in the differential count.

The inability to deliver cells to the site of challenge involves the paralysis of some function apart from the defective hemodynamics, since the shocked rabbits were challenged after the hemodynamic status was virtually normal.

We have already alluded to the leukotoxin obtained from the reversibly shocked rabbit 4 hours after transfusion. Because the early and rapid decline in the granulocyte count in shock is so much like the response of these cells to the injection of endotoxin, there is reason to suspect that a leukotoxin similar to endotoxin, at least with respect to this property, develops very early in shock. Pending the results of a current effort to isolate and identify such a toxin early in shock, it is relevant at this juncture to take note of our observation that whereas certain antibiotics given prior to shock prevented irreversibility to transfusion, the same antibiotics given shortly after inducing shock failed to do so,[2] in spite of the reasonable assumption that toxin does not form after their administration. In view of this, the inescapable inference from our general hypothesis is that the amount of toxin developed early in shock prior to giving an antibiotic, even if not added to as shock continues, must account for the development of irreversibility to transfusion. Since this irreversibility does not develop until after 3 or 4 hours of shock, we are compelled to make the further inference that a small amount of toxin formed early in shock can become a lethal dose if the sensitivity to toxin increases as shock continues. That this is true is evident from the results of sensitivity tests with a standardized intravenous dose of an *Escherichia coli* endotoxin.[15] A normal rabbit, weighing 2 to 3 kg., was killed by 10^{-1} mg., whereas another rabbit of the same weight and litter was killed by 10^{-6} mg. 4 hours after transfusion for shock of 2 hours' duration. Normal resistance to this toxin did not return until after 24 hours.

The progressive weakness of the antibacterial mechanisms can be explained either as a result of (1) the accumulation of toxins, which damage these mechanisms still further, thus creating a vicious circle; or (2) the deterioration of the tissues involved in this function, along with the deterioration of tissue functions in general, in proportion to the degree and duration of tissue anoxia due to deficient peripheral flow. The significance of tissue anoxia is indicated by the protective effect of the prophylactic administration of dibenzyline[16] or Thorazine,[17] which appear to act by diverting to the terminal circulation a larger-than-normal fraction of the total circulating blood volume, thus preventing the degree of tissue ischemia that occurs in response to hemorrhage in an animal with an unhindered vasoconstrictor mechanism. Another way of assessing the role of tissue anoxia in antibacterial defense is to reduce the tissue requirements for oxygen by hypothermia. In animals precooled to

28° C., hemorrhagic shock is better tolerated, the response to transfusion is excellent, and the survival time is prolonged from about 6 hours to 30 hours.[18] The delayed death of the animal must therefore be explained as due to the development of a lethal factor after transfusion. This factor was shown to be bacterial activity by the observation that if antibiotic therapy is started at the time of transfusion, all of these animals recover. Since such antibiotic therapy in normothermic animals is futile, the hypothermia must have provided some protection to the antibacterial mechanisms. This protection, though partial, is not inconsiderable for, when precooled dogs are challenged with intravenous bacteria during shock of 2 hours' duration, or in the normothermic state after transfusion, they recover, whereas, as already stated, corresponding normothermic dogs die.[19]

Further evidence that tissue anoxia injures the antibacterial mechanisms is the difference between the titer of properdin in a normothermic dog in hemorrhagic shock and a precooled dog in hemorrhagic shock. In the former, the titer falls precipitously within 2 hours and to a minimal level after several more hours.[20] In the latter case the titer is well sustained up to the time of transfusion (6 hours).[21] After transfusion and rewarming, the precooled dog's titer falls rapidly to minimal levels or to zero.

The following observation suggests a relationship between the properdin titer and the tolerance to shock: of 12 precooled dogs exposed to hemorrhagic shock for 6 hours, 3 died during the period of shock. The remainder were in good condition up to the time of transfusion. The 3 that died prior to transfusion had a properdin titer of 3 units or less prior to shock. The remainder had normal or near normal properdin titers.

Data on the relationship between the properdin titer and the shocked animal's resistance to infection remain to be provided. Relevant preliminary data are the following observations: the morphologic injury produced by exposure of granulocytes or macrophages to plasma from shocked dog is prevented if 0.04 ml. of this plasma is previously diluted with 0.16 ml. of normal plasma. This ratio is 0.16 to 0.04 when the normal plasma is fortified with 16 units of properdin per mil.[22]

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NUTRITIONAL AND GENETIC FACTORS IN THE NATURAL RESISTANCE OF MICE TO *SALMONELLA* INFECTIONS

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What *is* natural resistance? This is an odd question to raise in this publication, but there are some aspects of this question that need analysis before describing some of our experiments that, loosely interpreted, might fall into the category of answers. The analysis that I propose is philosophical and psychological and, if I begin with philosophy, it is because I believe philosophy rightly comes first, guiding us to appropriate experiments. The time for philosophy is at the beginning, so that we may end by confronting the natural world, and not the other way 'round, beginning with experiments and ending by confronting ourselves and one another.

First, "natural resistance" epitomizes a problem. What is this problem? Where are its beginnings? The problem has its seat, I believe, in our inability to incorporate into the already existing body of our theoretical understanding of infection the simple experience that a microbe that is pathogenic for a given species often proves to be without effect for a second. Here, before we have taken the first step toward understanding this experience, the problem has been "loaded." It has become "loaded" because, in epitomizing this experience, we all too quickly introduce the word "resistance," which hints of the kind of analysis that we anticipate, namely, that nonresponding species oppose our attempt to elicit disease. This opposition we find frustrating, for the mere fact that we have attempted to produce an infection clearly indicates our power-seeking motive to mastery. In the case of the susceptible species, where our theoretically directed operations result in infectious disease, we feel a sense of power and, to paraphrase Francis Bacon, that our power is knowledge. We feel that we understand. But when, with the mere change in host, the identical operations result in no disease, we feel frustrated, we feel opposed, we feel that something is "resisting" us and, by projection, we feel that the microbe, too, is being "resisted." With this outlook it is the most natural thing in the world to expect that an attempt to understand will generate a search for such items as are already in our theoretical framework of infection and that would be elements of opposition to microbial pathogens, that is, natural antibodies, bactericidins, virucidins, bacteriostatic entities, anatomical barriers, lysins, and the like. It may turn out that such a strategically oriented view may prove fruitful but, when one considers the psychological origins of the view that I have sketched above, I think that there is good reason to be less than enthusiastic and, I feel, there is some room for disquiet.

Permit me to start the analysis of the problematic situation all over again, and let us see whether we can avoid "loading" the problem with hints of what the answers should be. Then let us go to the natural world with some experiments and see if we can pose some unambiguous questions in the hope that the answers will bear the same stamp.

My analysis will be on 2 levels: (1) on the differences in susceptibility *between* host species, and (2) on the differences in susceptibility *within* host species. I begin with susceptibility, which is the basis of our awareness of infectious disease in the first place, and I define it as a capacity to respond to the microbic agent with the signs we label as disease. Now there are, for a given pathogen, responding and nonresponding species, or susceptible and insusceptible species. For these latter, instead of conjuring up forces of "resistance," which I think would be gratuitous, we are on better ground and show more philosophical parsimony if we admit that we do not understand, at this interspecific level, why the insusceptible species lacks the capacity to respond to the infectious agent. In another place[4] I have discussed the obstacles that prevent us, at this time, from making either a genetic or nutritional analysis of these interspecific differences. Suffice it to state here that technical difficulties block our analysis, and let us proceed to the more hopeful area of *intraspecific* differences in susceptibility to infectious disease. For this purpose I shall draw on a single, well-worked model of an infectious disease, namely, mouse salmonellosis. As a representative of its species, the laboratory mouse is notoriously susceptible to infection with *Salmonella typhimurium*, as laboratory-stock epizootics and infection experiments all easily attest. For identical doses of *Salmonella* introduced in the identical way, however, it is also true that differences exist between mice in the sense that an extremely varied response can be observed as experiences with this infection model accumulate, ranging from sudden and early death through various degrees of illness, with or without death, to complete survivorship. Here are our raw sense data: for with identical doses administered in identical ways, some mice die and some mice live. Why?

The first award for penetration into this problem, I think, goes to the geneticists[1, 7] who by inbreeding and selection showed that these differences, whatever their bases might be, could be manipulated by planned mating and assembled into stocks that were widely and predictably divergent in their response to the infectious agent. By tradition these stocks are labeled as "susceptible" and "resistant," but may I remind you that there is still no evidence that the "resistant" stocks embody any added something that the susceptible stocks lack. At the moment, to be rigorous, we must label these stocks as "insusceptible" and suspend our judgment, for the possibility exists that these insusceptible stocks *lack* something rather than *have* something. This is all to say that at the genetic level we face the same problem the physiological geneticist faces, namely, whether in a given instance the allelic difference is ascribable to a gain or to a loss. All we know with certainty is that a difference exists. That this difference can be arranged by appropriate breeding, and that the stocks thus produced can present us with this difference for study, reproducibly and at will, is an important, strategic contribution. All that would remain, it seems, is to examine these divergent genotypes and establish in just what phenotypic detail they differ. Unfortunately we do not know precisely what to look for, although this is not for lack of suggestions, some of which are included in this monograph. I do not propose now to make a case for or against any of these suggestions, but to direct your attention to a hitherto

unappreciated aspect of the host-pathogen situation that must be cleared away before our analysis can continue.

What is this new aspect? It is simply this, that intraspecific natural resistance is operationally dependent upon the structure, the composition of the testing pathogen population. In our hands, host differences in the mouse salmonellosis model are directly dependent upon this factor.[3] Briefly, a uniform, clonal population of virulent *S. typhimurium* kills *all* of these divergent genotypes we have just been discussing; a uniform clonal population of avirulent *S. typhimurium* results in survivorship of these same mouse genotypes; and it is only when we arrange a mixed population of virulent and avirulent *S. typhimurium* that we find sanity restored, with "resistant" genotypes surviving and "susceptible" genotypes succumbing. This experience I have summarized in FIGURE 1.

The Effect of Pathogen Population Composition on Host Survivorship

		Host genotype		
		Inbred, selected, resistant	Random-bred, (outbred) nonselected	Inbred, selected, susceptible
Pathogen genotype	Clonal virulent	Died!!	Died	Died
	Mixed virulent and avirulent	Survived	?	Died
	Clonal avirulent	Survived	Survived	Survived

FIGURE 1.

In the upper left-hand corner of this figure we see that the so-called "resistant" genotypes all died when infected with a uniform clonal population of *S. typhimurium*. Where is their resistance? That they are entitled to be regarded as different from the susceptible genotypes is evident from the test with the mixed population. It also irrevocably follows, however, that this treasured genotype that we have received from the hands of the geneticist cannot be regarded as resistant unless, at the moment of our inspection, we specify that the testing pathogen population is heterogeneous in its composition with respect to virulence. What we still have to account for, however, is the original success of the geneticist in selecting out these divergent host strains by the tests he conducted during this process, in which the specification of heterogeneity of the pathogen that I have advanced, as necessary and important, was never mentioned or appreciated. The reason for this was, historically, an automatic and built-in feature of the way the tests were conducted. The testing strain of pathogen was a carefully husbanded and conserved strain. Even if this test strain were uniform at the start, which it probably was not, we now know from direct experiments[2] that merely storing the stocks in the ice box on slants, as was the custom, would result, in the short space of weeks, in a heterogenous stock. The genetic experiments themselves, we know, were of necessity spread over many years. There is thus ample reason to believe that the important role of genetic heterogeneity of the pathogen in experiments in natural resistance is not a "Johnny-come-lately" but has been a silent and unappreciated factor from the very outset.

This role of the genetic heterogeneity of the pathogen population as the operational base of our awareness of host differences in the genesis of disease is not confined to the differences arranged by the genetics of the host. It is at the bottom of nutritional manipulation of infectious disease as well. When we add nutritional manipulations to the genetic analysis we can synthesize our experience by the chart presented in FIGURE 2.

Here we see that the nutritional manipulation of survivorship in an infectious disease is confined to that model in which a genetically heterogenous host is infected with a genetically heterogenous pathogen population. The nutritional difference we are examining here is the difference between a diet of natural food materials and a chemically defined so-called synthetic diet. The reasons for this choice have been described elsewhere,[5] and need not detain us at this time. The lesson we draw here, however, is that we have now added a second specification to our operations, that is, that in addition to the heterogeneous pathogen population in our infection model, we must also insist that the host be unselected and genetically heterogeneous. Here at last is the area in which we can now begin to untangle the effect of nutrition on survivorship in an infectious disease.

Thus far I have been emphasizing that all we have had in our hands is a manipulable difference in survivorship, first by genetics and now by nutrition, but I have pointed out, in the course of this paper, the reasons for suspending our judgment as to whether we should be looking for opposed forces or for lack of capacity to respond, which would be another matter entirely. Put in another way, is a greater survivorship due to an increased something, or to a

The Effect of a Natural (N) and a Synthetic (S) Diet on Survivorship Following Infection in Nine Different Genetic Circumstances

		Host genotype		
		Inbred, selected, resistant	Random-bred, (outbred) nonselected	Inbred, selected, susceptible
Pathogen genotype	Uniformly virulent	N - Died S - Died	N - Died S - Died	N - Died S - Died
	Mixed virulent and avirulent	N - Survived S - Survived	N - Survived ↑ Dietary effect S - ↓ Died	N - Died S - Died
	Uniformly avirulent	N - Survived S - Survived	N - Survived S - Survived	N - Survived S - Survived

FIGURE 2. From H. A. Schneider, in *Biological Foundations of Health Education*. 1950. Columbia University Press. New York, N. Y.

lack of something? The geneticist is unable to answer this question for us, but the nutritionist now provides us with the answer. I shall give you this answer at once. It is that increased survivorship can be achieved by *both* means, by addition *or* subtraction of host physiological entities. Thus, in mouse salmonellosis, survivorship can be increased by *subtracting* fat from the diet, and also by *adding* certain other items from the world of natural foodstuffs. Now we can come down off the fence and give operational definitions to the words that have been at the tips of our tongues, but which we have restrained ourselves from using and, as we now realize, for very good reasons. For our skepticism has been borne out by events. Improved survivorship in infectious disease, when host-arranged, may be due to the lack of something that we should now freely label a decrease in susceptibility, or this improvement may be due to the gain of something that we now freely label an increase in resistance. Let me briefly recapitulate to the point at which we have now arrived. In our analysis of the mouse-salmonellosis model we have become aware that differences in survivorship, whether arranged by nutritional or

genetic means, are operationally dependent upon a virulence-avirulence heterogeneity of the pathogen population. When this heterogeneity is removed and replaced by homogeneity, the host-arranged differences in survivorship disappear. A study of nutritional influences on these survivorship differences has shown that survivorship can be increased by either of 2 diametrically opposed operations, either by withholding fat or by adding a new entity as yet undefined. Based on these operations we have defined the first as a susceptibility factor and the second as a resistance factor. In other words, when we add something to the host physiology and survivorship goes down, we shall call that a susceptibility factor, and when we add something and survivorship goes up, we shall call that a resistance factor. Now the question arises, what is the chemical nature of these factors?

In my concluding remarks I shall confine myself to the resistance factor, upon which we have spent most of our time, and leave aside the investigations on the susceptibility factor, the study of which we have only lately resumed. In order to get on with the technical task of sorting out and identifying the new resistance factor from natural products, I beg your indulgence to describe briefly the assay on which this work is based. As I have stated, the arrangement of a nutritional difference in survivorship in mouse salmonellosis is operationally based on the peculiar procedure of infecting the mice with 2 strains of *S. typhimurium*, a virulent strain and an avirulent strain. In order better to understand this phenomenon, which has been dubbed the double-strain phenomenon, we have introduced, with the assistance of Norton Zinder, the use of genetic markers on the infecting bacteria. The avirulent strain is xylose nonfermenting, and the virulent strain has been given the distinguishing heritable marker of a xylose-fermenting capacity by the isolation of an appropriate mutant. This new virulent strain is as virulent as the parent strain. On appropriate, differential, xylose-containing media these 2 strains are easily identified and, after they have been introduced into the mouse, their separate kinetics of multiplication and their ultimate fate can be traced easily. The important bacteriological event, which is influenced by the diet of the host and which is correlated with survivorship, is the early suppression of the usual increase in numbers of the virulent salmonellae in the mouse. In a recent publication[6] the analysis is detailed by which we finally arrive at the stage where we substitute a bacteriological datum such as one presented to us by a measurement of the size of the virulent population in the spleen of a mouse at an empirically determined time. To spell this out, the assay now consists of measuring the virulent population in the spleen of 5 test mice, in the presence of the avirulent population, on the second day postchallenge with the virulent strain of *S. typhimurium*. If the spleen contains less than 100 virulent cells it is judged to be a positive response, that is, that particular mouse, if not sacrificed for 30 days, would have survived. A population of virulent cells greater than 100 indicates a nonresponding mouse, that is, that particular mouse would have died if not sacrificed for the 30-day test period. The procedure of counting black colonies on a plate is now the epistemic correlate, the actual sense-inspected data by means of which we infer the occurrence of the

event that excited our interest in the first place, namely, host-manipulated survivorship in an infectious disease. With this assay we can predetermine the frequency of ultimate survivorship in an infectious disease. With this assay we can detect the presence of survivorship-promoting activity, nutritionally supplied, by using 5 mice in a test with a total time elapse of 8 days. This substitution of a bacteriological datum for survivorship is an important and worrisome departure. That this datum is indeed linked to survivorship, our primary concern, in a firm 1:1 relationship is indicated in FIGURE 3. This correlation we find reassuring.

By the use of this assay we have been able to track down, for a considerable distance, the chemical entity from the world of natural foodstuffs that is responsible for this survivorship effect. The richest sources of the factor thus far found have been wheat, malted barley, and commercial dried egg white. From these sources the activity is extractable with methanol, and can be concentrated and purified in a schema such as that presented in FIGURE 4. Following chromatography on resin columns (IRC-50) and sublimation of the ammonium-formate buffer, active fractions can be further chromatographed on paper in a butanol-acetic acid-water system (R_f approx. 0.8) and eluted strip material subjected to paper ionophoresis. Ranging from pH 4 to pH

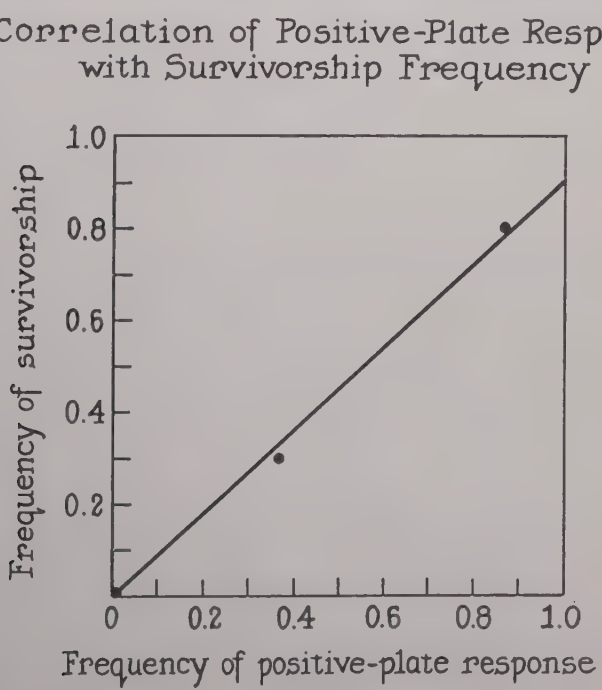

FIGURE 3.

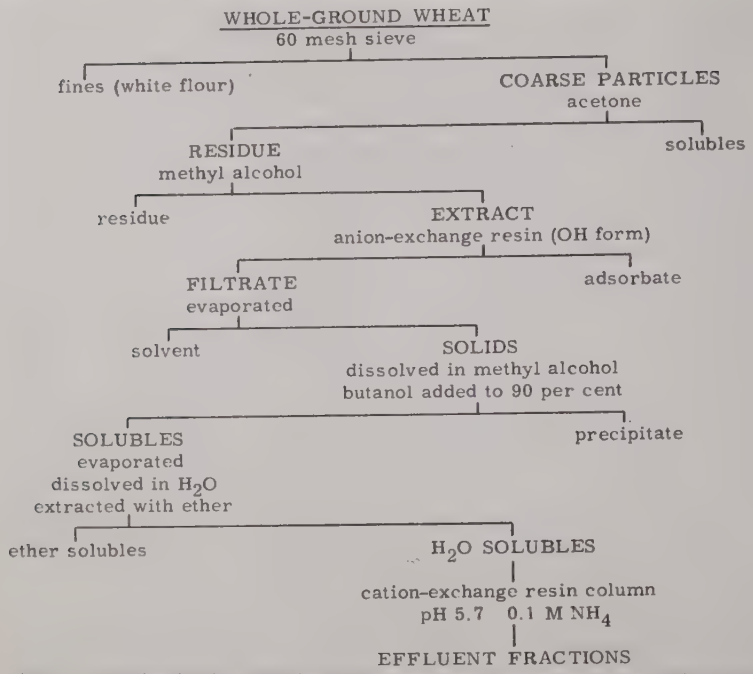

FIGURE 4. Fractionation of an active wheat for the mouse salmonellosis resistance factor. Active fractions are in capitals.

9, the activity zone showed no tendency to migrate in the electric fields applied. Activity is dialyzable, is destroyed by ashing, is stable to heating in aqueous systems around neutral pH, but is destroyed in 0.1N alkaline and 0.1N mineral acid. It is stable to ultraviolet light and purified solutions are spectroscopically empty in the ultraviolet region, possessing only a nonspecific end absorption. Concentrated solutions are yellow to brown. No positive clue has thus far been obtained as to chemical structure. All measurements of activity have been made by bioassay. The over-all concentration from a source such as wheat has been of the order of one millionfold, with an efficiency of about 20 per cent.

From the foregoing I believe that we must conclude that, in the natural world, there exist nutritional entities capable of enhancing natural resistance to infection as we have analyzed it here. These entities, which are probably distinct and different for each biological group of diseases, are apparently present in small amounts, and from an epidemiological viewpoint are probably but mere ripples on the deep ocean-swell of evolutionary and genetic adjustments between the host and pathogen species. In appropriately constructed models, however, they can be ascertained and, by concentration, their effects might be enhanced. This, if true, would be an important and strategically

different way to control infectious disease. What are the prospects? With this examination I shall conclude.

Using our double-strain inoculation assay in the mouse-salmonellosis model we have found[6] that the nutritional factor is not stored. When mice are placed on the basal synthetic diet, the effect of all previous dietary history is lost within 2 days. When the resistance factor is supplied, its effects are evident almost immediately and the factor can be withdrawn on the second postinfection day with the same ultimate beneficial result on survivorship as if it had been fed continually to the end of the 30-day observation period. We con-

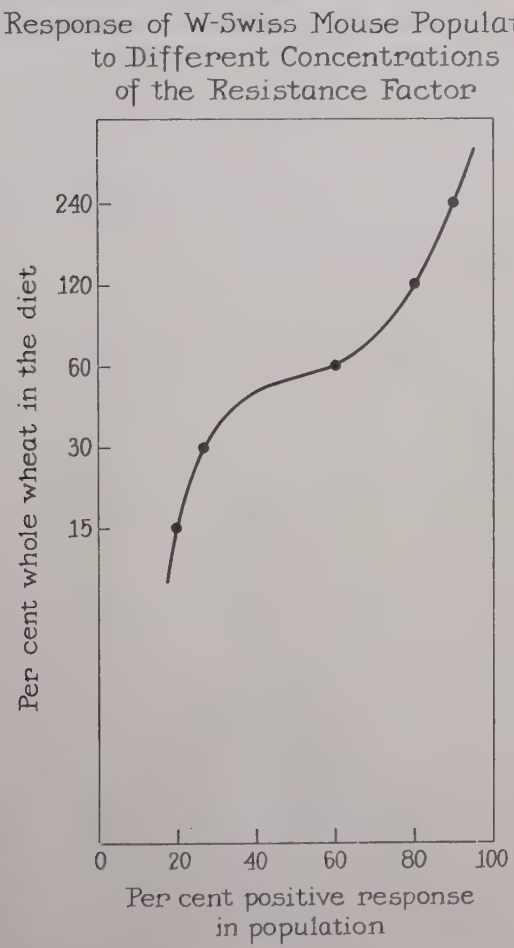

FIGURE 5.

clude that the nutritional resistance factor we have defined here is in a dynamic equilibrium in the host, and that its action in the early hours of the infection is decisive. All that follows, either death or survival, is a consequence of that decision.

Now what of the effect of concentration of this factor in the diet? How much do we have to add to the diet in order to protect how many more mice in the infected population? The dose-response curve is given in FIGURE 5. It will be noted that we obtain a typical quantal response in the shape of a sigmoid curve. It will also be noted that the ordinate is in logarithms of dietary concentration. This we interpret to mean that if we increase the dietary concentration *logarithmically* we can expect to protect more and more of our animals, for our mice are responding as a normally distributed population, as FIGURE 6 indicates. The origins of some of our troubles are now evident. In order to cope with the statistical problems of dealing with infection in these terms, we have had to embrace the world of infection *as it is*. In order to attain mastery we must be prepared to expend the effort to concentrate from the natural world certain powerful items that are present in low concentration and, when we are prepared to squander these items, we can construct a new dietary environment in which, without formal limit, we can embrace the world of infectious disease and bend it to our will.

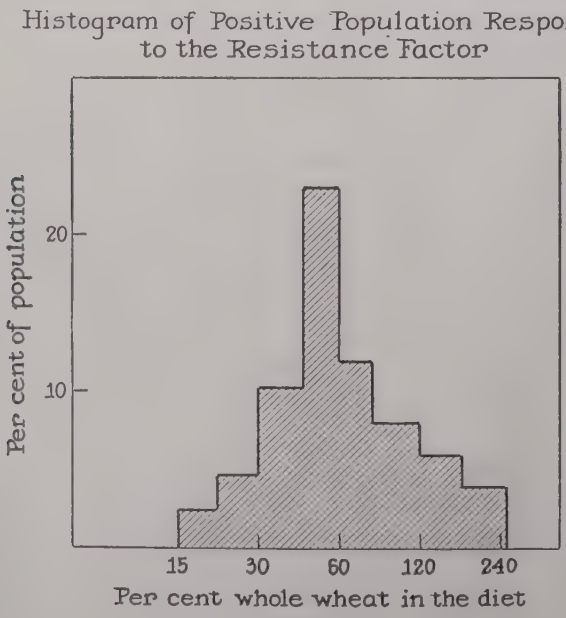

FIGURE 6.

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CELLULAR PRODUCTS AFFECTING THE ESTABLISHMENT OF BACTERIA OF DIFFERENT VIRULENCE

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In the search for host factors with significant influence on resistance and susceptibility, some attention must be given to the host's ability to act as a selective agent controlling the establishment of specific genetic types of a given pathogen. It has been suspected for many years that only specific and highly virulent types of bacteria tend to propagate in susceptible hosts, whereas relatively avirulent types may persist in resistant hosts (Hadley, 1937; Braun, 1953). With the rapid progress in the field of microbial genetics during recent years has come additional confirmation of the constant, spontaneous occurrence of antigenically altered mutants of different virulence in any microbial population of sufficient size. It has been demonstrated that such spontaneous mutants arise at rates that are typical and constant for a given strain, for example 1×10^{-7} per generation, but whether such mutants will outgrow their parental type will depend upon the existing environmental conditions. Thus, genetically altered cells of different virulence may arise either during the parasites' propagation *in vivo* or they already may be present within a genetically heterogeneous population at the time of the invasion of the host. The question then arises: To what extent is the ability of the host to influence the subsequent *in vivo* establishment of either highly virulent types or of less virulent types of a given pathogen causally related to its resistance or susceptibility?

As we shall see, we still are far from a satisfactory answer to this question. Quite a number of cases of striking correlations between selective effects and host resistance or susceptibility are known, however. These cases have encouraged, and continue to encourage, inquiries into the causal relationships involved, and they suggest the possibility of modifying such selective effects for therapeutic purposes.

I wish to cite briefly 2 examples to illustrate some of the observed relationships between selective effects and host susceptibility. First, let me cite the following example from our past work with *Brucella suis* (Kraft and Braun, 1952): After infection of susceptible hosts, such as guinea pigs, with a mixture of virulent S cells and relatively avirulent non-S cells, a rapid selection of virulent S cells occurs (TABLE 1). In contrast, infection of more resistant chicks with a comparable mixture of S and non-S cells results in a selective establishment of the less virulent non-S type (TABLE 1). A second example of relationships between selective effects and susceptibility is provided by studies on the increased virulence of *Salmonella typhimurium* in guinea pigs treated with threonine (Page, Goodlow, and Braun, 1951). Whenever the animals received an injection of 50 mg. of DL-threonine at the time of infection with a lethal dose of *S. typhimurium*, they died far more rapidly than similarly infected but untreated animals. It could be shown that this effect was due to the fact that the inoculum contained both threonine-susceptible *Salmonella* cells of

TABLE 1

COMPARISON BETWEEN SELECTIVE EFFECTS IN CHICKS AND GUINEA PIGS INOCULATED WITH S, M, AND R MUTANTS OF *BRUCELLA SUIS*. NUMBER OF CELLS INOCULATED INTRAPERITONEALLY INTO EACH ANIMAL: 2×10^9

Host	Inoculum: 51% M + 49% S % M recovered from spleens of individual animals after 1 week			Inoculum: 48% R + 52% S % R recovered from spleens of individual animals after 1 week		
Chick	99	93	32	66	98	92
Guinea pig.....	<0.01	<0.01	<0.01	<0.1	<1	<0.01

moderate virulence plus a few more threonine-resistant cells of far greater virulence. The 2 types were distinguishable by striking differences in their colonial morphology. In the threonine-treated animals, the more threonine-resistant, more virulent type established itself selectively, causing early death, whereas in untreated animals the propagation of the less virulent, threonine-susceptible type was favored.

Incidentally, these virulence-modifying effects of the amino acid were absent when threonine was injected into animals that had been infected with a homogeneous *Salmonella* population consisting of threonine-susceptible cells only. This phenomenon can be explained on the basis that there is insufficient time prior to death caused by the less virulent type to permit the selective establishment of the more threonine-resistant, more virulent cell type that arises at a relatively low rate (1×10^{-6}). If a few cells of the latter type are initially present, however, they will be selected immediately after infection of threonine-treated animals. These observations provide a striking parallel to earlier findings of Schneider (1946) regarding the necessity of a *heterogeneous* parasite population for the production of nutritional modifications of the susceptibility of mice to salmonellosis. The observations on the selective effects of threonine also suggest that normally existing or nutritionally modified differences in the amino-acid levels of tissues might affect the susceptibility of the host. It appears entirely possible that many previously observed effects of nutrition on susceptibility (Clark, 1950) might involve similar selective effects of the host upon a heterogeneous parasite population.

Virulence-enhancing effects also have been observed with another amino acid, namely D-alanine, in subsequent *in vivo* studies with *Brucella* species (Mika, Goodlow, and Braun, 1952). The repeated administration of alanine to guinea pigs infected with 1 $I.D._{50}$ of S *Brucella abortus* cells resulted in a significantly higher infection ratio. Tests on the cells recovered from these animals showed that the increased virulence was associated with the establishment of more alanine-resistant, more virulent S mutants *in vivo*.

In all of the previous examples, artificially modified selection *in vivo* had resulted only in the establishment of mutant cells with *greater* virulence. It was hoped that a better understanding of the nature and mode of action of the factors controlling population changes towards avirulence *in vitro* may eventually permit controlling the establishment of *less* virulent types *in vivo*. Considerable information on such *in vitro* factors has been collected in recent years, a good deal of it in studies with *Brucella* species that were carried out in

collaboration with my former associates Goodlow and Mika. Most of the accumulated data have been the subject of extended discussions elsewhere (Braun, 1952; 1953). Let us merely recall here that it was demonstrated that population changes involving the establishment of non-S mutants in initially S cultures are promoted by the accumulation of particular amino acids in the culture medium. These amino acids accumulate as end products of the cells' metabolism, and high levels of certain amino acids, especially D-alanine, were found to inhibit the growth of the parental S-type cells without interfering with the propagation of spontaneously arising less-virulent non-S mutants. Similar inhibitory effects of accumulating amino acids, giving rise to population changes, were noted in studies with a variety of bacterial species. The nature of the inhibition was studied in detail only with *Brucella* species, however, where the results indicated that the inhibition of S cells by the metabolic product alanine involves an interference with pantothenic acid synthesis (Mika *et al.*, 1954). Later data have suggested that these inhibitory effects, and thus the associated population changes, are greatly dependent upon oxygen tension (Braun *et al.* 1956; R. Altenbern).

One phenomenon observed in these *in vitro* studies should be cited here in more detail because it illustrates how specific nutrient substrates of the parasite may influence the subsequent selective establishment of specific mutant types. It was found that when D-asparagine is the sole nitrogen source in initially S *Brucella suis* broth cultures, alanine accumulates in the medium and favors the establishment of more alanine-resistant, spontaneously arising R mutants (TABLE 2). When L-asparagine is used as a nitrogen source in the culture medium, however, valine and not alanine accumulates and favors the establishment of valine-resistant M mutants (TABLE 2). The implications of such observations for the possible influence of specific host and tissue factors on the selective establishment of specific mutant types of pathogens should be apparent without further discussion.

At this point it was tempting to speculate that the information on selective effects of naturally occurring compounds might be applicable to bacterial population events *in vivo*, that is, to population changes that may occur following the penetration of the parasites through the initial host defenses. Subsequent investigations with experimentally infected animals (Braun *et al.* 1951; Mika, Goodlow, and Braun, 1952; Simon, Redfearn, and Berman, 1955) re-

TABLE 2

POPULATION CHANGES AND METABOLISM IN INITIALLY SMOOTH BRUCELLA SUIS CULTURES MAINTAINED IN A SYNTHETIC MEDIUM WITH D- OR L-ASPARAGINE AS THE SOLE SOURCE OF NITROGEN

Configuration of asparagine	Percentage of variants on the following days					Accumulating amino-acid metabolites
	5th	8th	12th	16th	20th	
L	0	0	52M*	52M	41M	Valine
D	16 R†	55R	84R	90R	97R	Alanine

* M = Mucoid.
† R = Rough.

vealed that the information on selective effects of amino acids observed in test-tube cultures, that is, in a closed system, cannot be applied readily to population changes *in vivo*. The simple reason for this difference is the fact that the *in vivo* environment more nearly represents an open system rather than a closed system, preventing the necessary accumulation of high amino-acid levels. Thus non-S types of *Brucella* do not tend to establish themselves in most tissues of guinea pigs after prolonged infection with S-type cells. It was noted, however, and confirmed in concurrent studies by Jones and Berman (1951), that less virulent non-S types of *Brucella* can be recovered from closed abcesses of guinea pigs infected with S cells. The amino-acid levels in these abcesses proved to be high and thus may represent the exceptional closed *in vivo* environment in which selective effects comparable to those noted in test-tube cultures can take place.

Several years ago we described a selective serum factor, the SS factor (Braun, 1949), that was strikingly correlated with host resistance and susceptibility to brucellosis. Population changes from S to non-S occurred in broth cultures in the presence of serum or of serum globulins from *resistant* hosts, but such population changes involving the establishment of less virulent cell types were suppressed in the presence of serum from *susceptible* hosts. The subsequent elucidation of factors controlling population changes revealed that this serum factor exerts its effect through its influence on the rate of accumulation of alanine in the culture medium. Since, as just mentioned, these selective amino-acid effects appear to play a major role only in a closed environment, it has become obvious that the SS factor has little or no causal relationship to natural resistance or susceptibility. This selective serum factor now must be placed into the steadily increasing category of host factors that merely are curiously correlated with different states of susceptibility.

An entirely different selective factor capable of promoting the establishment of virulent types in initially avirulent bacterial populations has been recognized in recent *in vitro* studies. It was found that a breakdown product of bacterial desoxyribonucleic acid (DNA), obtained after exposure of DNA to the enzyme desoxyribonuclease (DNase), will kill relatively avirulent cells without inhibiting the propagation of virulent cells (Braun and Whallon, 1954). Thus, DNA + DNase added to initially non-S cultures of *Brucella* species will promote the rapid establishment of spontaneously arising virulent S mutants (TABLE 3). Actually, this selective activity is not associated with the initial breakdown product of DNA that is obtained after exposure to the depolymerizing enzyme DNase but, as determined with the help of filtrates from supplemented S cultures, the active component results from the action of S cells upon this initial DNA breakdown product. Thus avirulent non-S cells are *not* inhibited by DNA + DNase until a few S cells occur spontaneously in the growing population, or until a filtrate from S cultures exposed to DNA + DNase is added (TABLE 4). This phenomenon has permitted the consistent duplication *in vitro* of the type of bacterial population change that characteristically occurs within susceptible hosts, namely, from non-S (avirulent) to S (virulent). The effects of this selective factor have been identical regardless

of whether the DNA added to the cultures was isolated from S or non-S *Brucella* cells, from *Escherichia coli* cells, or from pneumococcus cells. Also, this selective effect of a DNA breakdown product favoring the multiplication of the virulent cell types apparently is not restricted to *Brucella* cells. Comparable effects have been obtained with pneumococcus cultures containing initially 99.9 per cent R and 0.1 per cent S cells (W. Firshein). Under the influence of the DNA breakdown product such cultures changed to 95 per cent S and 5 per cent R within 24 to 48 hours (TABLE 5). Thus both in *Brucella* and pneumococcus cultures the newly discovered factor selectively favors the establish-

TABLE 3

THE EFFECTS OF DNA, DNASE, AND DNA + DNASE UPON POPULATION CHANGES OF INITIALLY M CULTURES OF *BRUCELLA ABORTUS* GROWN IN BUFFERED BEEF-EXTRACT BROTH

Medium	Extent of population changes after	
	5 days	9 days
Control	<1% S	<1% S
DNase (75 γ/ml.)	2% S	4% S
DNA (150 γ/ml.)	3% S	6% S
DNA + DNase	33% S	60% S

TABLE 4

THE EFFECTS OF FILTRATES FROM 48-HOUR-OLD S CULTURES, WITH OR WITHOUT DNA + DNASE, UPON POPULATION CHANGES OF INITIALLY M CULTURES OF *BRUCELLA ABORTUS* GROWN IN BUFFERED BEEF-EXTRACT BROTH

Added to medium:	Extent of population changes after 6 days:
Filtrate from S cultures	<1% S
Filtrate supplemented with DNA + DNase after filtration	3% S
Filtrate from S cultures grown in presence of DNA + DNase	41% S
Controls	<1% S

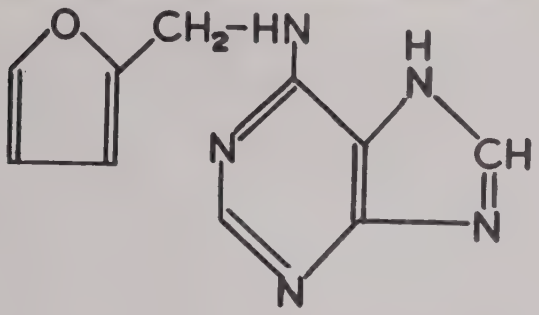
TABLE 5

THE EFFECTS OF BREAKDOWN PRODUCTS OF DNA ON POPULATION CHANGES OF PNEUMOCOCCUS CULTURES IN BRAIN-HEART INFUSION BROTH

Inoculum: 99% R, 1% S (A 66)

Medium	Per cent S after 48 hours
Controls	5
DNA (Pneumococcus) + DNase*	95
DNA (*Brucella*) + DNase	90
6-furfurylaminopurine, 0.03 γ/ml	65
6-furfurylaminopurine, 1 γ/ml	12
6-furfurylaminopurine, 3 γ/ml	7
Autoclaved deoxyadenosine, 250 γ/ml	35
Unautoclaved deoxyadenosine, 250 γ/ml	0

* 150 γ/ml. + 75 γ/ml.



6-Furfurylaminopurine (Kinetin)

FIGURE 1.

ment of virulent cell types, even though in 1 case (*Brucella*) the virulent cells are characterized by the presence of a protein surface antigen and in the other case (pneumococcus) by the presence of a polysaccharide capsule.

The active DNA breakdown product has proved to be relatively heat-stable and alcohol-precipitable. It is unaffected by high pH (10.2 for 48 hours) but it is affected by exposure to low pH (2.6 for 48 hours). Its activity could not be duplicated by any of the commercially available purines, pyrimidines, nucleotides, or nucleosides that have been tested.

Within the last few months it has been possible to isolate from the sonic extract of *Brucella* cells (either S or non-S cells) an alcohol-precipitable, protein-containing fraction that specifically antagonizes the just-described selective effects of the DNA breakdown product.

A compound with known chemical structure has been found that possesses selective activity similar to that obtained after addition of DNA + DNase to *Brucella* or pneumococcus cultures, and it also inhibits avirulent cells without affecting virulent cells, thus promoting population changes from non-S to S. The active compound is 6-furfurylaminopurine, also called kinetin, which recently has been shown by others (Miller *et al.*, 1955) to enhance cell proliferation of plants when used in conjunction with 3-indoleacetic acid. Kinetin*, if used in sufficiently high dilutions, proved to be active both in the *Brucella* and pneumococcus systems (TABLES 5 and 6). It has been reported that kinetin can be obtained from deoxyadenosine after autoclaving at pH 4.0 for 30 minutes (Hall and deRopp, 1955). As shown in TABLES 5 and 6, autoclaved deoxyadenosine does possess selective activity, whereas unautoclaved deoxyadenosine tends to counteract such effects. It still remains to be determined whether the selectively active product from bacterial DNA is identical with or similar to 6-furfurylaminopurine, and whether such selective substances play any significant role in supporting the establishment of virulent cell types *in vivo*. The

* Kindly supplied by E. R. Squibb and Sons, New York, N. Y.

TABLE 6

THE EFFECTS OF BREAKDOWN PRODUCTS OF DNA ON POPULATION CHANGES OF *BRUCELLA ABORTUS* IN BUFFERED BEEF-EXTRACT BROTH

Inoculum: M cells

Medium	Per cent S after 12 days
Controls	1
6 furfurylaminopurine, 0.1 γ/ml	40
6-furfurylaminopurine, 1 γ/ml	15
6-furfurylaminopurine, 10γ/ml	1
Autoclaved deoxyadenosine, 250 γ/ml	12
Unautoclaved deoxyadenosine, 250 γ/ml	0
DNA + DNase*	60

* 150 γ/ml. + 75 γ/ml.

isolation of a bacterial cell fraction with antagonistic activity against the DNA + DNase effects has been mentioned above. It will be interesting to test whether this antagonist also might aid in preventing or retarding the establishment of virulent bacterial types in susceptible hosts.

The foregoing data show that with the help of cellular products we now can control, to a considerable extent, *in vitro* population changes of certain bacterial pathogens either in the direction of increased or decreased virulence. This means that we can supply environmental conditions that will selectively favor the establishment of either highly virulent mutants or of mutants with reduced virulence. Comparable selective effects probably occur *in vivo*. Thus, different levels of amino acids shown to be capable of exerting selective effects upon bacterial populations *in vitro* may exist in different hosts and different tissues, and cellular destruction yielding breakdown products of DNA with selective activity probably can take place at sites of propagation of the parasite *in vivo*. It will require continued investigations, however, to determine more accurately the actual extent to which such selective effects of the host upon changes in the parasite population may contribute generally to the phenomena of resistance and susceptibility.

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NONSPECIFIC DEFENSE REACTIONS IN BACTERIAL INFECTIONS

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Enhancement of Staphylococcal Lesions by Various Modifiers

Shock. A few years ago, when working on shock in relation to local infection of the skin,[1] Janet Niven and I made the unexpected observation that started the work presented here. We measured the change in infectivity of staphylococci in guinea pigs subjected to 2 to 3 hours of dehydration shock. In a typical experiment, a standard dose of staphylococci was injected into the skin of the subject every hour for 8 hours, and soon after the beginning of this period the animal was shocked with intraperitoneal hypertonic glucose. The infectivity of the cocci was estimated from the diameter of the lesion after 24 hours. The uniform final lesion diameters resulting from the hourly injections in control animals proved that the dose of cocci had remained constant throughout the experiment. In shocked animals the lesions initiated during shock were, after 24 hours, substantially larger and more severe than those in the controls. The increase in severity corresponded to a fiftyfold increase in the dose of cocci, and we attributed this enhancement of infection to a hypotensive ischemia of the skin during shock, with a consequent reduction of the antibacterial substances, including leukocytes, arriving at the infective site from the blood.

The unexpected observation was that lesions 1 to 2 hours old when shock began were insusceptible to enhancement, although at this stage they still had 20 hours of progressive development to pass through before attaining the maximum size and severity.

Three inferences can be made from this experiment. First, since the shock enhanced the lesions fiftyfold, the defense mechanisms inhibited by the shock must, in the control animal, have led to the death or removal from the site of all but one fiftieth of the inoculum. Second, these particular defense mechanisms operated within the first 2 to 3 hours of the infection. Third, during this short period, the ultimate size of the lesion was determined to some extent.

Adrenalin. Too much happens in shock for us to be certain that the local enhancement results solely from a peripheral ischemia. An analogous but entirely local effect can be produced by the injection of adrenalin at the site of infection. Two μg., in our standard injection volume of 0.1 ml., are sufficient for a local ischemia that lasts 1 to 2 hours and that, on passing off, leaves the tissues almost as resistant as they were before the injection.[2] This dose enhances *Staphylococcus aureus*, for example, about twelvefold (FIGURE 1). This enhancement is evident from the horizontal shift in the plot of mean lesion diameters against the logarithm of the dose of the cocci injected with and without adrenalin. When the adrenalin is injected into a series of established staphylococcal lesions aged 0, 1, 2, and up to 6 hours, only the 0-hour lesions are enhanced (FIGURE 2), and the final diameters of lesions more than

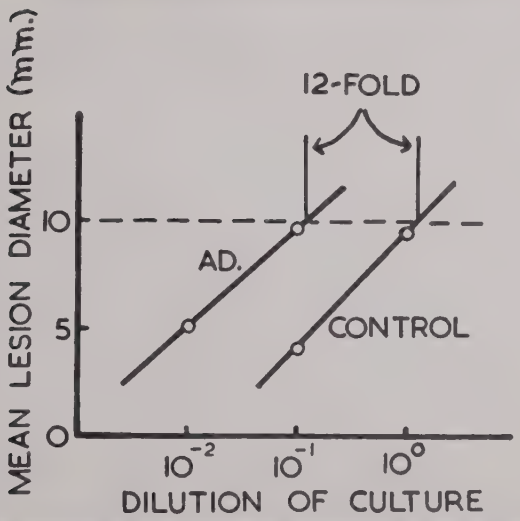

FIGURE 1. The enhancement of *Staphylococcus aureus* infections in the skin by 2 μg. adrenalin. The dose of bacteria is plotted logarithmically, and the horizontal distance between the 2 response lines, measured at the 10-mm. lesion diameter, is log 12. (IN FIGURES 1 to 6, each point represents the mean of at least 4 lesions, each in 1 guinea pig. The lesion diameters are measured at 24 hours.)

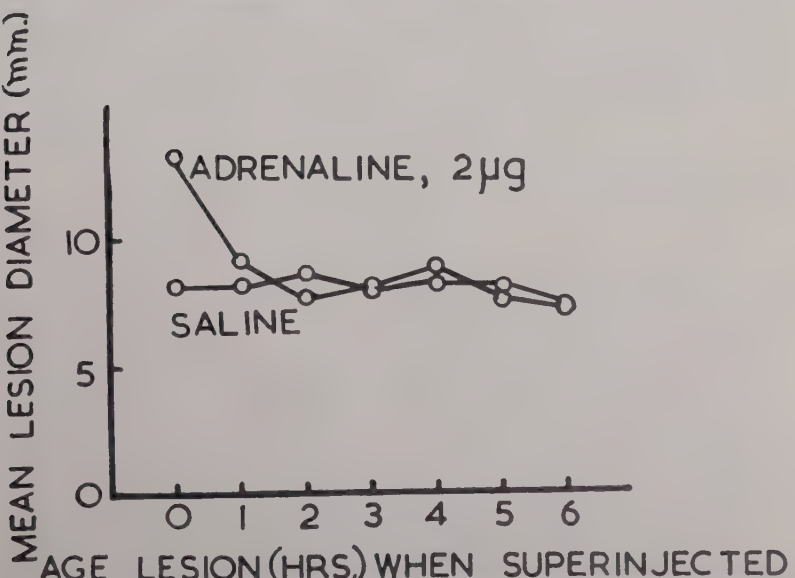

FIGURE 2. The decreasing susceptibility of *Staphylococcus aureus* lesions to enhancement by 2 μg. adrenalin, with increasing age of the infection. The adrenalin was "superinjected" through the needle puncture made when the bacteria were injected. Saline was superinjected into the control lesions. Decisive period about 1 hour.

1 hour old when the adrenalin was injected are no greater than the control lesion diameters. That is, as far as the defenses modifiable by local ischemia are concerned, the maximum development of the lesion is decided within the hour.

Liquoid. Staphylococcal infection is also enhanced by the anticomplementary and anticoagulant compound sodium polyanethol sulphonate (Liquoid). This is a much more brutal inhibitor of defenses than adrenalin because, in effective doses from 50 to 100 μg., its local enhancing activity is much greater and lasts, although with diminishing effect, from 4 to 5 hours. In 1 test, when Liquoid was injected together with the bacterial suspension, it enhanced activity two hundred and fortyfold (FIGURE 3). Nevertheless, susceptibility to enhancement diminishes with the age of the lesion, and Liquoid had substantially no action on 5-hour-old lesions (FIGURE 4). In these staphylococcal examples we have certain defense reactions that we can *define* as the reactions inhibited, respectively, by simple ischemia and by an anticomplementary substance. The value of these defenses in each case can be expressed numerically as the inverse of the degree to which the modifying agents enhance the infection. Thus if Liquoid enhances one thousandfold in terms of the dose of bacteria that produces a standard effect, then the Liquoid-inhibitable defenses are worth a thousandfold-kill to the animal. (It should be noted that Liquoid does not appear to enhance infection by inhibiting coagulation in the tissues because heparin enhances few skin infections, and in anticoagulant doses equipotent with 50 μg. Liquoid, has less than one thirtieth of the inhibiting potency of Liquoid on complement, on the bactericidal power of the blood, or on the chemotactism of microphages.) The de-

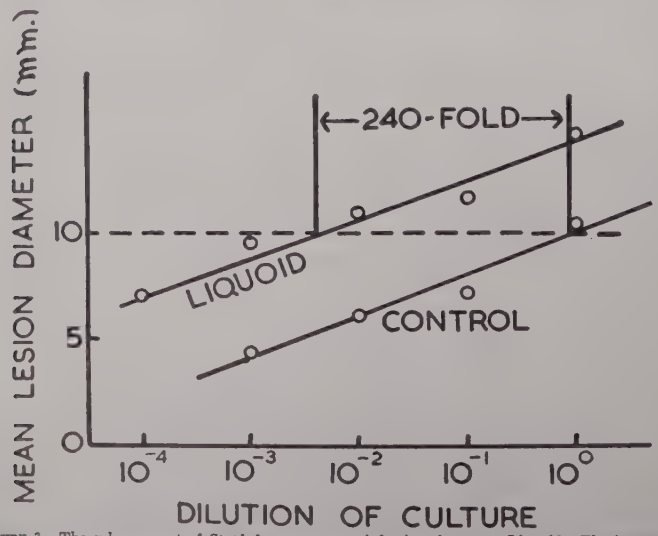

FIGURE 3. The enhancement of *Staphylococcus aureus* infections by 50 μg. Liquoid. The increased infectivity of the cocci (two hundred and fortyfold) is measured as in FIGURE 1.

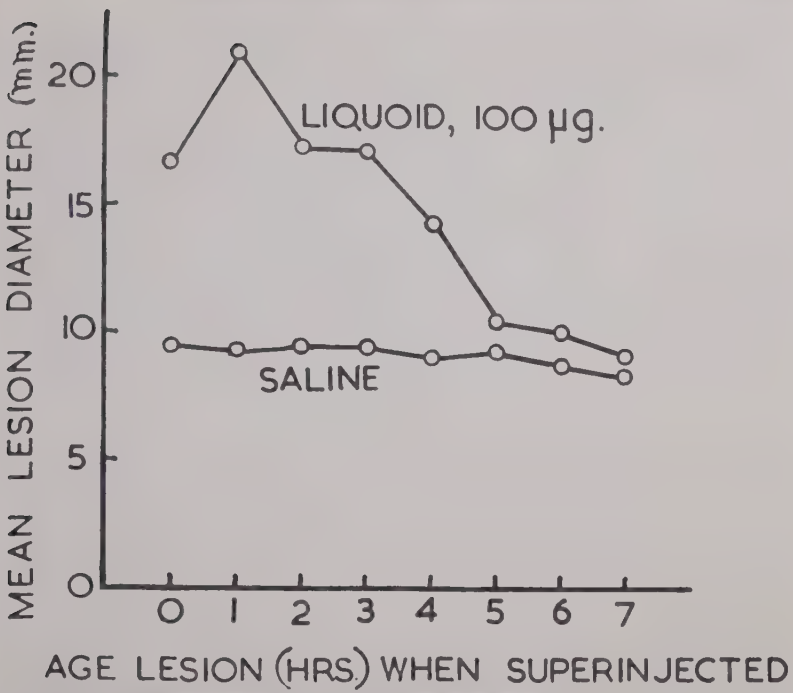

FIGURE 4. The decreasing susceptibility of *Staphylococcus aureus* lesions to enhancement by 100 µg. Liquoid, with increasing age of the infection (FIGURE 2). Decisive period 5 hours.

fenses, moreover, act soon after the introduction of the infecting bacterium, and are effective only for a short period—less than 4 hours—during which the ultimate size of the infective lesion is decided.

The Numerical Value of Local Defenses Against Various Pathogens

Generalizations about infective diseases from a study of a single example of them are notoriously risky and misleading, and we therefore set out to discover the efficacy of these reactions in different kinds of infection, and whether, as with staphylococci, these reactions occur within a short decisive period. We used 9 different species of bacteria, including *Staphylococcus aureus*. They were chosen, first, not because they were natural pathogens, but primarily because in reasonable doses they would produce measurable skin lesions whose maximum diameters, when plotted against the logarithm of the dose, yielded dosage-response curves such as those shown in FIGURES 1 and 3. They were chosen, in the second place, to represent as wide a variety as possible of pathogenic species. Not all the bacteria yielded linear responses, and the dose-response lines in control and test series were not always parallel. For numerical estimates of change in infectivity, we worked, therefore, with doses producing mature lesions within the 7 to 12 mm. range, and made our comparisons

at the 10 mm. level of response. For the most part, however, over a thousand-fold or more dose range, the parallelism and linearity were good enough to justify generalizing about the numerical estimates of defensive value made at the 10-mm. level.

Some of the 9 pathogens were gram-positive, some gram-negative (TABLE 1). The skin virulence, in terms of numbers of washed viable organisms producing a 10-mm. lesion, was relatively high in *Listeria monocytogenes* and the 2 corynebacteria, moderate in the *Streptococcus*, the *Staphylococcus* and *Pseudomonas pyocyanea*, and low in the *Clostridium welchii*, *Escherichia coli* and *Proteus vulgaris*. Excepting the corynebacteria, which eventually killed the guinea pigs by intoxication, none of the bacteria produced generalized infections in the doses employed. We were therefore exploring defenses sufficient to prevent generalized disease but insufficient to stop the local development of inflammatory and sometimes necrotic lesions that in all cases reached a maximum size in from 24 to 36 hours and then slowly regressed. Enhance-

TABLE 1

Bacterium	E.D.$_{10}$
Strep. pyogenes Group C	2×10^6
Staph. aureus	2×10^7
C. diphtheriae (*milis*)	5×10^4
C. ovis	5×10^4
L. monocytogenes	1×10^4
Cl. welchii	5×10^7
E. coli	1×10^9
Pr. vulgaris	5×10^8
Ps. pyocyanea	5×10^6

The pathogens used to test the local-skin defenses of the guinea pig. The E.D.$_{10}$ (effective dose) is the approximate number of washed viable bacteria in a 0.1-ml. injection volume producing final lesion diameters of 10 mm.

TABLE 2

Infecting agent	Defenses inhibited by					
	Adrenalin		Liquoid		Shock	
	P	DP (hrs.)	P	DP (hrs.)	P	DP (hrs.)
Strep. pyogenes Group C	0.1	4	0.006	2	*Mod.	2
Staph. aureus	0.1	2	0.005	4	Small	4
C. diphtheriae (*milis*)	0.1	4	0.1	4	(-ve)†	—
C. ovis	0.5	1	0.1	3	Large	3
L. monocytogenes	0.4	2	0.05	1	Mod.	5
Cl. welchii	0.00001	3	0.025	3	Mod.	3
E. coli	0.0001	1	0.12	1	Mod.	4
Pr. vulgaris	0.001	1	0.0006	5	(-ve)	—
Ps. pyocyanea	0.01	2	0.0001	3	Small	3

The killing power of infected skin evaluated as the estimated proportion of the inoculum (*P*) destroyed by the defenses that are inhibited by 2 μg. local adrenalin, 50 to 100 μg. local Liquoid, and 2 to 3 hours dehydration shock, and the decisive period (*DP*) in hours during which these defenses operate.

* Mod. = moderate.

† This symbol (-ve) = infection *depressed* by shock.

ment, and the time relations of susceptibility to enhancement, by adrenalin, Liquoid, and dehydration shock were determined by the method already described for *Staphylococcus aureus* and, as before, the enhancement by adrenalin and Liquoid is translated into the proportion (*P*) of the original inoculum that *apparently* survives the defenses inhibited by these 2 modifiers (TABLE 2). That is, an x-fold enhancement is entered as the survival in the unmodified infection of only one xth part of the inoculum. *Corynebacterium ovis* and *Listeria monocytogenes* survive adrenalin-inhibitable defenses rather well, but even so their infectivity is reduced by 50 and 60 per cent, and the survival of the remainder is smaller, ranging from 1 in 10 to 1 in 100,000. Liquoid-inhibitable defenses, as we might expect of reactions defined by a more brutal inhibitor, are generally more effective. The shock-inhibitable defenses were not measured with any precision, but ranged from about 0.1 (moderate) to 0.0005 (small).

The Decisive Period in Defense

Whatever their magnitude, however, the defenses explored in this way are far from negligible and, without exception, they operate within 5 hours of the introduction of the bacteria. The decisive periods cited in TABLE 2 are *maximum* periods, found as the result of at least 2 and, in many cases, 3 or 4 determinations, in each of which the mean diameters were obtained from 4 to 8 replicate lesions in a total of 4 to 6 animals. Twenty-three of the 25 estimates are 4 hours or less, and the general average for the decisive period is 2.8 hours. The shock technique, it should be noted, was not applicable to *Corynebacterium diphtheriae* and *Proteus vulgaris*, because shock substantially *decreased* the lesion sizes in both infections, a phenomenon we are not yet able to explain but that nevertheless serves as a useful warning against generalization about these effects.

These are results with *local* skin lesions. It also can be shown that these early reactions may be decisive for more remote *systemic* consequences of local infection. For example, my colleague A. C. Dutton[3] infected mice with subcutaneous doses of the Group C *Streptococcus* that killed in about 5 days. The death rates in batches of 10 mice were greatly increased when adrenalin or Liquoid was injected into the same site immediately after the cocci, but were like those of the control animals when the drugs were injected into subcutaneous lesions 4 hours old.

I want to stress that these defenses, judged by the period of maximum susceptibility to enhancement were, on the whole, most effective during the first 90 minutes of infection, and declined steadily in efficacy to the end of the decisive period. Their widely varying effect on the 9 different pathogens may well express basic differences in the type of infection. The important common feature, however, is the consistent limitation of the decisive period to the first few hours. It may be objected that the definition of the period is artificial and its significance dubious, because larger doses of the modifiers, or other more powerful modifiers, might enhance infections more than 4 hours old, as indeed sometimes happens. The objection, however, is not relevant to the argument because the quite substantial defensive value of the reactions

I am discussing was determined by the same concentration of modifier as that used to establish the decisive period itself.

The relation of these early defenses to natural nonspecific resistance is clear enough. These defenses appear, in fact, to *be* nonspecific. They act on bacteria introduced into tissues immediately beneath the epithelium, that is to say, on pathogens in a situation analogous to the primary lodgment of a natural infection of the animal through some breach in its epithelial surface. They are obviously capable of diminishing the infectivity of recently introduced bacteria to an extent that, were they acting on a natural invader, the primary lodgment would be made much less dangerous and, in many cases, diminished even to the point where it would be unable to produce even a local infection. It is indeed probable that some experimentally observed changes in resistance, supposed to be due to effects that are sustained throughout the course of the infection, are due solely to modifications at the primary lodgment of the infecting dose.

None of the pathogens we used had the startling virulence we find in some laboratory infections, where 5 to 10 bacteria are ultimately fatal. But nature, if we are to judge by morbidity and case-fatality rates, seldom works with 100 per cent infecting or 100 per cent killing doses. Only in raging epidemics of diseases such as plague or cholera, or in sporadic diseases such as glanders or rabies, is the body attacked by an $L.D._{100}$. The attack is usually made with doses that prove to be well below the $I.D._{20}$ infectious dose and the $L.D._{20}$. For this reason we feel justified in regarding these moderate experimental pathogens as not-too-remote models of the natural bacterial hazards to which a healthy animal is subjected.

The Decisive Period in Relation to Antibiotic Therapy

Before leaving the decisive period, the point is worth noting that it turns up in another, perhaps more familiar, context. Penicillin and streptomycin were given in single intravenous or intraperitoneal doses that diminished infections to about the same degree that these infections were enhanced by the standard doses of adrenalin and Liquoid (since the tests were finished in 24 hours, the delayed toxicity of penicillin for the guinea pig can be ignored). Thus a single intravenous dose of 12,000 units of streptomycin per kg. diminishes the infectivity of contemporaneously injected *Pseudomonas pyocyanea* about forty-fivefold (FIGURE 5). In this instance the comparison is made at the 7.5 mm. lesion diameter. When the intravenous dose of streptomycin is given to an animal bearing *Ps. pyocyanea* lesions that are 4, 3, 2, 1, and 0 hours old (FIGURE 6), however, only the 0-hour lesions display the same susceptibility. One-hour and 2-hour lesions are slightly larger, but the bacteria appear to be wholly insusceptible in the lesions that are 3 hours old when the drug is given. General experience of chemotherapy would lead us to expect that the older the infection, the less effective the drug. It is striking, nevertheless (TABLE 3), that with the single exception of the continuing susceptibility of the streptococcal infection for 8 hours and more, the decisive period in all the infections susceptible to the dose of antibiotic employed was usually 3

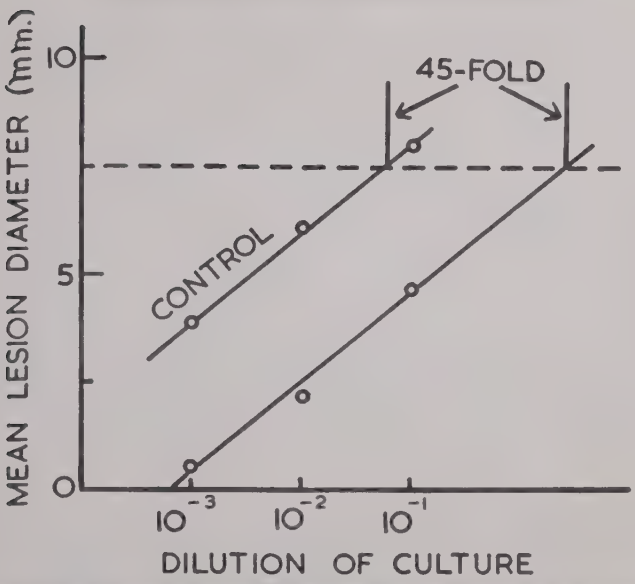

FIGURE 5. The forty-fivefold suppression of *Pseudomonas pyocyanea* infection by the contemporaneous intravenous injection of 12,000 units of streptomycin per kg., compared with infections in untreated animals.

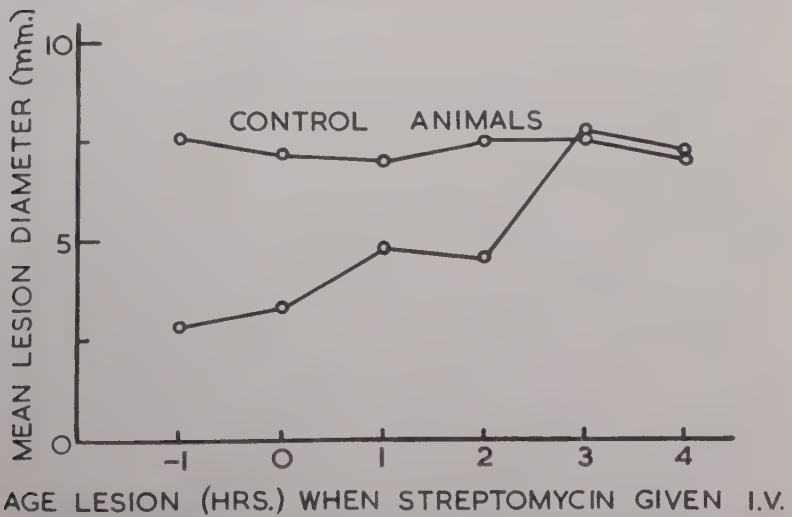

FIGURE 6. The decreasing susceptibility of *Pseudomonas pyocyanea* to a single intravenous injection of 12,000 units streptomycin per kg., with increasing age of the infection, compared with lesions in untreated control animals. Decisive period 3 hours.

TABLE 3

Infecting agent	Bacteria inhibited by			
	Penicillin		Streptomycin	
	P	DP (hrs.)	P	DP (hrs.)
Strep. pyogenes Group C	0.1	3	0.02	>8
Staph. aureus	0.07	3	0.004	5
C. diphtheriae (mitis)	0.05	3	0.01	4
C. ovis	0.007		0.005	5
L. monocytogenes	—		—	
Cl. welchii	0.1	3	—	
E. coli	—		0.1	3
Pr. vulgaris	—		0.01	3
Ps. pyocyanea	—		0.02	3

The depression of skin infectivity of various pathogens by a single intravenous injection of 10,000 units penicillin G/kg. and 12,000 units streptomycin/kg., evaluated as the estimated proportion of the inoculum (*P*) surviving the bactericidal effect of the antibiotic, and the decisive period (*DP*) in hours during which the infections are modified by the circulating drugs. The negative sign indicates infections not modifiable by the antibiotics in the doses used.

hours and never more than 5 hours. It is also striking that the bacteria in the lesion should be so completely insusceptible, in spite of the fact, described later, that the blood flow through the vascular bed in the lesion is quite unimpaired at this stage. These antibiotic observations are relevant chiefly as additional evidence that blood-borne modifiers that in this case are small-molecular and readily diffusible apparently do not affect the primary lodgment after 4 hours.

The Sequence of Early Inflammatory Events in Bacterial Lesions

At the end of the decisive period, the defenses appear to have been mobilized, and the battlefield limited thereby to a determined region. In contending that the outcome is determined, I do not imply that the battle is over, because a region that at 4 hours is mildly hyperemic in the next 20 hours becomes highly indurated and even necrotic. Nor do I contend that other events in the tissues could not modify the outcome after 4 hours, but such events would be of a different kind from the minimal modifications that we have imposed on the lesions, and would presumably act upon a different complex of defenses.

The next problem found in these indubitable early defenses is their relation to the classical tissue reactions during infection. The local bactericidal powers may reside wholly in the tissues, they may be activated by substances from the blood, or they may be blood elements that arrive at the primary lodgment as a result of tissue reactions induced by the infecting agent. The defensive significance of the various inflammatory phenomena, and of proven bactericidal elements of the blood, both humoral and cellular, are commonplaces in our textbooks of pathology. As with many other commonplaces, however, the truth of some of them has been established largely by repetition, and the truth of others is often established by appeal to a rather shaky principle, namely, that if a reaction is associated with successful defense, it is probably

defensive. The vascular changes of inflammation—vasodilatation, increased stickiness of the capillary endothelium, increased permeability of the capillary wall to large molecules, and diapedesis of leukocytes—can certainly be induced at speeds that qualify them as participants in early defense. When care is taken to introduce bacterial pathogens so well washed that their immediate irritant effect is reduced to a minimum, however, the classical process of inflammation is found to start some time after the early defenses have come into play. J. F. Burke and I[4] have explored the state of tissues infected by this method with respect to 5 characters determined in lesions of ages ranging from 0 to 5 hours or more. These are as follows:

(1) Increased capillary permeability, indicated by the exudation from the blood of the vital dye pontamine blue. This is observed directly in the skin of the living animal and recorded as lesion diameter.[5]

(2) Increased stickiness of the capillary endothelium, indicated by the adherence of circulating saccharated iron oxide to the inner walls of the blood vessels. For this purpose the animal is killed 1 hour after intravenous injection of the oxide,[6] and the iron converted to a visible Prussian blue by treatment of the skin with acid ferrocyanide. The diameter of the blued area is recorded.

(3) Diapedesis of leukocytes, indicated by the degree of tissue leukocytosis in stained sections.

(4) Thrombosis of the blood vessels, indicated by the interruption of the fine vascular plexus that is revealed in normal skin by the intra-arterial injection of India ink[5] at a pressure similar to that in the large vessels of the animal.

(5) Thrombosis of lymphatic vessels, indicated by interruption of the lymphatic plexus, revealed by intralymphatic India ink. Technical difficulties and the peculiarities of the lymphatic plexus precluded our examining the skin of the trunk in this way, and these tests were made on analogous lesions in the skin of the ear, where the plexus is readily demonstrable by microinjection.[7]

In many respects, the 9 test infections responded alike to these 5 tests.

It is convenient to deal first with the tests of blood and lymphatic vessels. The results with all the 9 pathogens were entirely negative up to the fifth hour. In the skin of the trunk, bearing lesions that in 24 hours become highly indurated and often necrotic infections 7 to 10 mm. in diameter, there was no interruption whatsoever of the blood vascular bed, either during its filling with ink in the living animal, or detectable by low-power microscopy of injected, fixed, and cleared preparations of the whole lesion. In the ear, infections were initiated in the fine anastomotic lymphatic plexus near the marginal veins by doses of bacteria that would produce 4 to 6 mm. indurated lesions in 24 hours. In all cases the infected plexus filled exactly as a normal plexus does, provided the lesions were not more than 5 hours old. After 5 hours, occlusion occurred in both blood and lymphatic vessels at the center of the lesions destined to become necrotic in 24 hours.

All the pathogens induced an immediate increase in permeability and sticki-

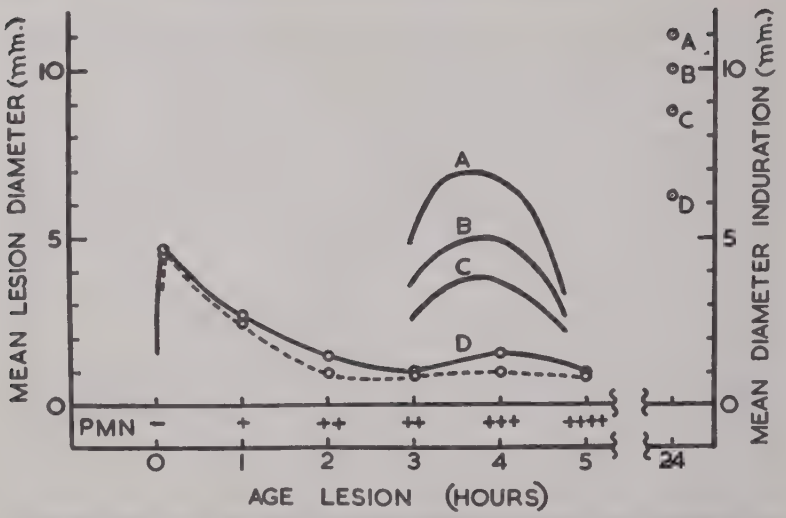

FIGURE 7. The vascular permeability to circulating pontamine blue (continuous line), the stickiness of the vascular endothelium for circulating saccharated iron oxide (dotted line) and the degree of tissue leukocytosis (*PMN*) in *Listeria monocytogenes* lesions of various ages. *A*, *B*, *C* and *D* represent the secondary permeability peaks and the final lesion diameters with 4 decreasing doses of the bacteria. The curves with *Corynebacterium diphtheriae* were similar.

ness that disappeared during the first hour. All induced a substantial secondary rise in these 2 qualities, starting after the second hour, attaining its maximum in the third to the fourth hour, and declining again by the fifth hour. Tissue leukocytosis usually began around the larger vessels lying on the panniculus carnosus toward the end of the first hour, became generalized in 3 to 4 hours, and increased steadily to the sixth hour. With *Corynebacterium diphtheriae* and *Listeria monocytogenes*, the secondary rise was pronounced only with initial doses that produced relatively large lesions at 24 hours. Thus with *L. monocytogenes* the dose producing the insignificant rise depicted in curve *D* of FIGURE 7 produced a 6 mm. lesion at 24 hours. A pronounced rise occurred after larger doses (curves *A*, *B* and *C*). In each case the final lesion diameter was from 2 to 2.5 times that indicated by maximum dye exudation at 3.5 hours. With *Proteus vulgaris*, the secondary increase in permeability was large, but here the final lesion diameter from graded doses of bacteria was consistently smaller than that of the dye exudation at 3.5 hours, the ratio of early to late diameters being about 10:7.

The Decisive Period in Relation to Changes in Vascular Permeability

The other 6 infections were alike in their vascular history, which is summarized schematically in FIGURE 8. Here the curves for stickiness are omitted, since they closely follow those for increased permeability. The secondary rise and fall in permeability is of particular interest, because its maximum diameter

in all 6 infections was about equal to the maximum induration displayed at 24 hours in FIGURE 8. Moreover, when the inoculum was smaller than that exemplified, the curve fell to zero at the fifth hour, and the final lesion had no necrotic center. With the larger inocula, the area of increased permeability had contracted by the fifth hour to a constant diameter that in each case proved to be about equal to the diameter of the central necrotic region at 24 hours. With all 9 pathogens, therefore, the physiopathological history of the vascular system indicates a decisive period similar to that inferred from a study of infectivity. Thus the final region of maximum induration can be predicted from the permeability state of the blood vessels at 3½ hours, and the region of the tissues destined to become necrotic is that in which at 4 to 5 hours the vessels have been so damaged that they can no longer recover their normal low permeability to the dye. The ratio of the lesion-diameter of early permeability change to that of final induration and necrosis was consistently about unity in 6 infections and, as already noted, was about 10:7 with *Proteus vulgaris* and 1:2.5 with *Corynebacterium diphtheriae* and *Listeria monocytogenes*.

Vascular thrombosis, as such, plays no part in this determination of lesion size, since none occurred until the sixth hour, and then only at the center of the lesions. The rising curves for thrombosis and induration in FIGURE 8 are to be regarded as rough indications only, since lesions from 6 to 20 hours old were not studied in any detail.

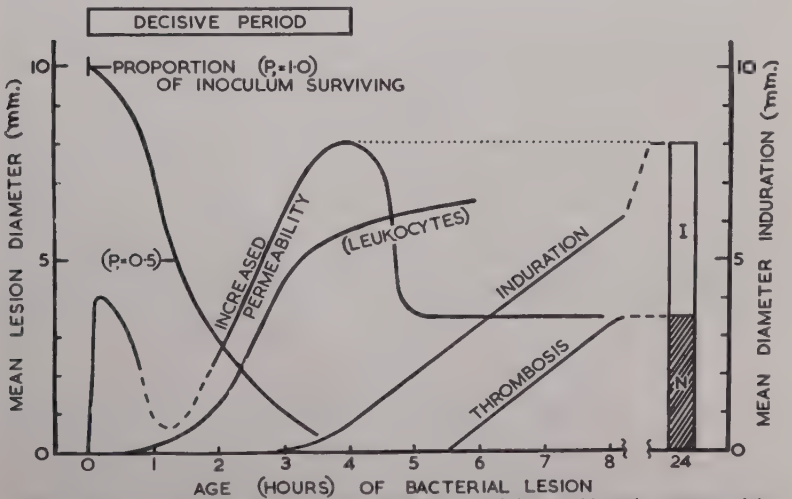

FIGURE 8. Synoptic history of vascular reactions to the primary lodgment of bacteria, common to infections with *Streptococcus pyogenes*, *Staphylococcus aureus*, *Corynebacterium ovis*, *Clostridium welchii*, *Escherichia coli* and *Pseudomonas pyocyanea*. Increase of vascular permeability, induration, thrombosis of blood and lymphatic vessels, and final induration (*I*) and necrosis (*N*) in lesions of various ages are indicated by lesion diameter. No ordinates are given for tissue leukocytosis, and the top of the "leukocytes" curve corresponds to maximum leukocytosis. *P* is an average curve of the estimated proportion of the inoculum surviving the tissue defenses (TABLE 2), in terms of unity, the dose of bacteria inoculated.

The Relation of Antibacterial Defenses to the Inflammatory Sequence

The secondary increase in permeability is accompanied by diapedesis. We are not yet sure whether diapedesis continues after the secondary decrease in permeability. If so, we may have to revise the common view that increased permeability is a necessary condition for diapedesis. A little more can be said about the primary and secondary peaks. The first resembles the permeability change induced by single local injections of histamine, histamine liberators,[5] and serum permeability factors.[6] The increase is small with well-washed bacterial suspensions, and large with less well-washed ones, and may therefore be due to small-molecular products from the culture adsorbed to the bacterial surface. It may indeed be wholly artificial, a consequence of using a substantial volume of a suspension as inoculum, and due to circumstances perhaps far removed from those of a natural primary lodgment. The second peak, in its magnitude and time relations, resembles the rise and fall that J. M. Elder and I[8] found to be induced by certain clostridial exotoxins, and may represent the point at which the surviving bacteria of the primary lodgment begin to multiply or, perhaps, begin to release toxic endosubstances. The noteworthy fact about both peaks is the subsequent restoration of normally low capillary permeability. There is a similar restoration after local injections of histamine and related permeability factors, and this restoration is associated with increased resistance to further action of permeability-inducing substances.[5] If the decrease in permeability at the fifth hour proves also to be associated with an increased resistance of the endothelium to permeability factors, in this case the presumably continuing intoxication by the invading pathogen, and if permeability increase has, in fact, a defensive value, it is possible that the outcome of the infection may be decided at the fifth hour because the blood vessels have become incapable of any further reaction of this kind.

The outstanding feature of the synoptic history of the infective lesion in FIGURE 8 is the association of maximum killing or removal of the primary lodgment (the curve *P* indicates the estimated surviving proportion of an average inoculum) with nothing more than a temporary increase in vascular permeability that occurs in only a small central portion of the area that ultimately will become the mature infective lesion. This increase, moreover, as we have seen, may be the unavoidable consequence of the experimental technique and without a parallel in natural infections. The outstanding defect of this synoptic history is the deduction of the *P* curve by an indirect argument from inhibition of defense reactions, and not from viable counts of survivors. There are many unsolved technical problems about making such counts in skin lesions that are too numerous to discuss here, although the few successful counts we have made are consistent with the killing curve that I have drawn. If the indirect argument is accepted, however, we must conclude that the substantial defenses of the decisive period are either inherent in the tissues or result from a temporary flow of humoral factors from the blood.

The pin-pointing of these factors is not easy. The exudation of plasma bactericidins is not the complete answer. It is indeed difficult to prove that any form of exudation is beneficial, because the results of artificial stimulation

of exudate on local infection are very equivocal and vary widely with the infecting bacterium. The biochemical and physicochemical circumstances of the primary lodgment, as well as the antibacterial properties of the tissues themselves, clearly demand investigation. The possibility that the early kill is in fact rapid removal of the bacteria from the infected site[7] cannot be dismissed, although viable counts suggest that the proportion of bacteria removed in this way is not great. On the other hand, the *in vitro* bactericidal power of fresh skin tissues has so far proved negligible.

Conclusions

These are some of the problems arising from the discrepancy between the sequence of the classical inflammatory reactions and the reactions in terms of which I have defined a decisive period in the history of the primary lodgment. That some such events must take place has for a long time been implicit in what is known of experimental infections. Nevertheless, if my interpretation of the time-course of enhancement is correct, I think that our observations add something to the general biology of early infection and defense.

First, these observations indicate a means of giving a numerical value to the early defenses and of assaying the effect of modifiers.

Second, these data show that the investigation of the infections mentioned and their experimental modification can be limited to local lesions during their first 3 hours of life. It is thus possible to make not only a topographical but a functional distinction between, on the one hand, the reactions that determine whether a pathogen shall get a lodgment in the body and, on the other hand, the reactions that determine the fate of an already established infection.

Third, the facts reported here define a stage in defense that fairly can be called nonspecific, a stage where nonspecific resistance can be investigated, uncomplicated by any consideration of antibody immunity.

Fourth, this report may perhaps provide a model for the investigation of all kinds of newly occurring lodgments of bacteria, including secondary lodgments like the metastatic lesions in the various organs of the body that arise in the course of generalized infection.

Finally, the definition of the decisive period indicates what we think is an important and rather neglected field of study, although the possible functions of the probable factors in that field are, in the present state of knowledge, still largely matters for speculation.

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EFFECT OF COMPONENTS OF THE TRICARBOXYLIC-ACID CYCLE ON BACTERIAL INFECTIONS*

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In order to establish the kind of tactical problem with which investigators in the area of natural resistance to infections are often confronted, assume for the moment that the survival time of an infected susceptible host, previously subjected to a set of experimental manipulations, is significantly shorter than the survival time of a control group of the same host. How is one to proceed in the laboratory to gain some insight into the mechanisms responsible for this change in resistance to infection? For the purposes of the report to follow, the notion that greater susceptibility is the result of a reduced resistance will be implied even though the potential fallacy of the reasoning, as emphasized so clearly by Schneider (1951), is recognized.

When confronted with a situation of this type it is possible to postulate a number of potential explanations, all based on confirmed experimental evidence. For example, the shorter survival time could be the result of an impaired cellular defense, a depressed humoral defense, both natural and induced, or it could be due to various combinations of these factors. The final analysis inevitably becomes complicated and exceedingly difficult to circumscribe with any assurance. The investigator is faced with a bewildering array of uncertainties because most of the important body defenses against bacterial infections cannot be quantitatively evaluated.

Determinations of phagocytic indices yield data of some reliability in experienced hands of 1 step, and 1 step only, in 1 group of cells of importance in the cellular defense. In cases in which this 1 step is normal, may it be concluded that the whole cellular defense is normal? Obviously not. Similarly, antibody titers may be semiquantitatively evaluated to give some idea of the ability of an animal to respond to antigenic stimulation, but this by no means yields the complete picture of the humoral defense status of the animal. Even if all such possible tests were made, the picture of an animal's defense against bacterial infection would not be complete.

To compound the picture further, it is self-evident that any viable infectious agent, such as pathogenic bacteria, must grow and reproduce in and at the expense of the host organism. Regardless of whether the pathogen is intracellular or extracellular, the host cells and the pathogen must require at least some, if not most, of their nutriments in common. A priori, it would seem that competition might exist between the cells of host and parasite for those molecules they both need for growth and maintenance. Is it not theoretically conceivable that, in this sphere of interaction between the 2 species of cells, events may occur to alter the course of a disease, even in the absence of a specific defense change that makes itself manifest in profoundly altered survival times

* This study was supported at Bryn Mawr College by funds provided under Contract AF 18(600)-511 with the United States Air Force School of Aviation Medicine, Randolph Air Force Base, Texas.

(Dubos, 1954)? Some experimental evidence related to these problems will now be examined.

Methods and Results

Animals subjected to the stress of simulated altitude are more susceptible than control mice to *Salmonella typhimurium* infection, as revealed by a statistically significant reduction in survival time. In these experiments, infections were administered intraperitoneally in the form of a saline suspension of 250,000 cells of an 18-hour brain-heart infusion-broth (Difco*) culture at time zero, with the results shown in TABLE 1. The shorter survival time of the altitude mice is statistically different from that of controls at the 1 per cent level. Similar results have been obtained with similar groups of mice infected intraperitoneally with *Diplococcus pneumoniae* (Berry, 1956). The altitude mice infected with this organism have a significantly shorter survival time than control mice. Highman and Altland (1955) have also reported that rats kept at simulated altitude develop more severe valvular lesions of the heart and other symptoms of bacterial endocarditis as a result of *Streptococcus* infection than do ground-level rats. With both *Salmonella* and pneumococci the difference in survival time between the experimental and control groups is not particularly dramatic, but it is consistent and reproducible. The question as to why it occurs and what change in defense, if any, has intervened to render the altitude-exposed animal more susceptible naturally arises.

A variety of tests have been carried out, as reported by Berry and Mitchell (1953a), to detect altered mechanisms of defense, but in all cases the data were not significantly different for the 2 groups of mice, including the ability of the reticuloendothelial system to sequester intravenously injected thorotrast. This last finding assumes some significance if we accept the evidence of Gordon and Katsh (1949) that normal hormonal stimulation from the pituitary-adrenal axis is necessary for normal reticuloendothelial activity. Such a test reveals, however, only 1 aspect of reticuloendothelial-system behavior. In effect, we have, with altitude-exposed mice, an example of animals with a changed susceptibility to infection without any specifically detected change in a mechanism of defense to account for it. To be sure, all body defenses were not and could not be evaluated, but at least certain capacities of the major defenses were given consideration. The possibility presented itself that in these animals some type of biochemical interaction between host and parasite cells might contribute to the observed findings. This concept was in line with the fact that mice comparably exposed to simulated altitude are more *resistant* than normal control mice to influenza A virus infection (Berry, Rubinstein, and Mitchell, 1955). The literature contains a considerable body of evidence suggesting that agents interfering with the synthetic capacities of host cells render the animal more resistant to not only influenza viruses but to other viral agents as well (Ackermann, 1951; Ainslee, 1952; Mogabgab and Horsfall, 1952; and others).

It was this line of reasoning that led to the testing of the effect of several inhibitors and intermediates of the tricarboxylic-acid cycle on the course of the

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TABLE 1

AVERAGE SURVIVAL TIME IN HOURS OF MICE EXPOSED TO HIGH ALTITUDE AND CONTROL MICE

Group	Number of mice	Average survival time in hours
Mice exposed to high altitude	40	42
Control mice	46	60

TABLE 2

EFFECT OF INHIBITORS AND INTERMEDIATES ON SURVIVAL TIME OF MICE INFECTED WITH *SALMONELLA TYPHIMURIUM*

Compound injected	Survival time in hours of mice injected with	
	Compound listed	Saline-control group
Malonate 8 × 1 mg./gm.	10	84
Sodium arsenite 3 × 6 μg./gm.	13	94
Sodium succinate 8 × 1 mg./gm.	21	95
Sodium citrate 8 × 0.5 mg./gm.	31	95
Fluoroacetate 2 × 8 mg./kgm.	40	87

infection in mice infected intraperitoneally with *S. typhimurium* (Berry and Mitchell, 1953b, 1953c; and Berry, Merritt, and Mitchell, 1954). The average survival times of groups of mice given sublethal amounts of different compounds is shown in TABLE 2. These data leave no doubt as to the greater susceptibility of the animals receiving certain of these compounds. Note in the left-hand column of the table the number of injections that were given and the dosage of each substance indicated under the name of the compound. The first injection was always given immediately after the animal was infected, and the remaining injections were given thereafter at intervals of 1 hour, except for fluoroacetate, where the second injection was given 6 hours after the first, and for arsenite, where the third injection was given 1½ hours after the second. In some instances only 5 mice were used in a group, but each experiment was confirmed, in most instances, by repeated tests. Even with a sample of 5 mice, when all of the experimental group die before the first in the control group, the statistical probability of this having occurred by chance alone is 1 in 100, according to the rank-order test of White (1952). For this reason large numbers of animals are not necessary to establish the validity of the observation.

With other bacterial pathogens similar results were obtained, as seen in TABLE 3. Note that with *Proteus morganii*, *Staphylococcus aureus*, and *Klebsiella pneumoniae* all of the control mice surived an infection that was uniformly fatal in the presence of certain inhibitors or intermediates. Note also that activity of a compound in 1 infection is no guarantee of its effectiveness in another. This suggests that a specific interaction occurs between the type of metabolic alteration and the ability of the pathogen to exploit it to its own advantage.

Since, in mice infected with *S. typhimurium*, the minimum survival time was obtained with a series of sublethal injections of malonate, this compound was selected as a type example for additional studies. It became of primary importance to know whether the infected mice surviving for such comparatively brief periods of time in the presence of malonate were actually dying of the infection or of a combination of infection and poisoning. This is obviously not an easy question to answer. It was reasoned that if the number of viable pathogens at the time of death could be determined in both control and malonate-injected mice there would then be some basis for deciding whether death was due to the infection. To this end it was established that reliable counts on SS agar could be made of a whole mouse homogenate prepared in a Waring blender (Berry, 1955). A known dilution of the carcass, based on weight, was prepared using normal saline as diluent, and plates of SS agar were inoculated in quadruplicate. Table 4 shows the average number of *S. typhimurium* found per gm. of mouse in control mice at the time of death and the number found in mice infected and given a series of injections of malonate. As before, 1 mg. of malonate was given per gm. of body weight every hour for a total of 8 injections. It is apparent from a comparison of the figures obtained in the 2 groups that there is no significant difference in the number of pathogens present at death. The total number of bacteria per mouse can be found by multiplying the number per gm. by 15, the average weight of the carcass minus skin, feet, and tail. From this evidence it is permissible to infer that the difference in survival times, 140 hours versus 30 hours, seems to be dependent upon the dif-

Table 3

Mean Survival Times of Mice Infected with Different Pathogens and Given a Series of Injections of Inhibitors and Intermediates of the Tricarboxylic-Acid Cycle

Mice infected with	Average hours of survival of mice injected with				
	Malonate	Succinate	Citrate	Fluoroacetate	Saline
Streptococcus pyogenes	7	12	12	30	12
Proteus morganii	6	20	survived	survived	survived
Staphylococcus aureus	6	survived	16	10	survived
Corynebacterium kutscheri	65	53	42	38	72
Klebsiella pneumoniae	18	24	survived	16	survived
Diplococcus pneumoniae	43	41	51	35	65

Table 4

The Average Number of Bacteria at Death in Mice Injected with *Salmonella typhimurium* and then Given Injections of Either Malonate or Isotonic Saline

Experimental conditions	Saline-injected mice	Malonate-injected mice
Number of animals	8	7
Average number of bacteria per gm. of mouse carcass	9×10^6	11×10^6
Average survival time in hours	140	30

ference in time required for an apparently constant lethal population of the pathogens to be reached within the mouse.

The question next arises as to why the *S. typhimurium* are able to reproduce so much more rapidly in mice given malonate than in control mice. Various possibilities exist. The difference could be due to 1 of 2 major causes. Either important body defenses of the animal are impaired by the action of the inhibitor and, by inference, by injections of intermediates of the tricarboxylic-acid cycle such as citrate, succinate, and others, or else the bacteria are provided with a nutritive environment that favors their more rapid reproduction.

The latter possibility is favored by the following evidence. The uptake of intravenous thorotrast is unaltered by the usual series of malonate injections in mice, as seen in TABLE 5. Thus, 10 hours after administering the thorotrast, the animals were sacrificed, their organs were removed, desiccated, and powdered, and their thorotrast content was determined with the aid of a Tracerlab scaler with a thin-walled Geiger tube.

The ability of blood neutrophiles of rats to ingest bacteria is unaltered by malonate injections under the same conditions that greatly reduce survival times of rats infected with *S. typhimurium*, as seen in TABLE 6. Because of the uncertainty in identifying ingested cells of *S. typhimurium* inside rat neutrophiles, due to confusion with granulations within the cells, a suspension of *Staph. aureus* was used in evaluating the per cent of active phagocytes. The numbers in parentheses represent the number of separate determinations from which the averages were derived. Note also that the malonate dosage, 1 mg. per gm. of body weight, was the same as that used for mice. Uninfected rats injected with this quantity of malonate survived.

TABLE 5

THOROTRAST CONTENT OF SELECTED ORGANS OF MICE INJECTED WITH EITHER MALONATE OR SALINE

Organ	Thorotrast content (in mg.) of organs of mice given	
	Saline (18)	Malonate (16)
Liver	13.4	12.0
Spleen	13.7	11.7
Lungs	1.2	1.0

TABLE 6

PER CENT OF RAT-BLOOD NEUTROPHILES SHOWING INGESTION OF *STAPHYLOCOCCUS AUREUS* FROM RATS INJECTED WITH EITHER ISOTONIC SALINE OR MALONATE, AND SURVIVAL TIMES OF INFECTED RATS UNDER SIMILAR CONDITIONS

	Saline injected rats	Malonate injected rats (1 mg./gm. of rat)
Per cent of phagocytes active	87—(11)	86—(12)
Mean survival time of rats infected with *Salmonella typhimurium*	116—(12)	28—(12)

TABLE 7

NUMBER OF *SALMONELLA TYPHIMURIUM* REMAINING VIABLE WITH TIME IN WHOLE SERUM FROM RATS INJECTED WITH SALINE OR MALONATE OR IN SERUM TO WHICH ZYMOSAN WAS ADDED

Time in hours	Number of bacteria in serum from rats given		
	Saline	Malonate	Zymosan added to serum
0	11,100	12,500	12,400
1	2,500	2,000	19,800
2	4,100	3,800	79,300
3	4,300	3,800	255,000
4	5,100	3,900	796,700
5	8,900	5,800	2,077,000

In addition, the ability of blood serum from rats injected with malonate to reduce the viable count of *S. typhimurium* is no different from that of control rats, while zymosan added to control sera neutralizes this ability. This is seen in TABLE 7. The bacterial suspension was added to the serum contained in small tubes kept in a 37° C. incubator at all times except when samples were withdrawn for dilution counts. The malonate and saline injections were administered to the rats at hourly intervals prior to the time the animals were bled from the heart in order to collect the serum. Pooled samples from 3 animals were used for each test, and the figures presented are the averages of 3 separate determinations. The zymosan was added to the serum according to the directions of Pillemer *et al.* (1953) for neutralizing the action of properdin. The bactericidal power of the serum appears to be unaltered by malonate and, inferentially, by the properdin system. Since these are merely a few of the potentially important defenses found to be unimpaired by malonate injections, it is always possible that other defense mechanisms are inhibited.

This problem may be approached somewhat indirectly in an effort to gain some insight into the possible role played by other body defenses. Since it is a well-known fact that heat-killed suspensions of endotoxin-producing pathogenic bacteria, when administered in sufficient numbers, are lethal for susceptible animals, it becomes important to inquire into the influence of the number of killed pathogens on the number of viable pathogens at death. The question can be posed in another way. Let us assume that an experimental group of mice is more susceptible than a control group because of an impaired defense mechanism. This would mean that during the course of the disease fewer of the causative bacteria would be destroyed in the bodies of the experimental group of animals. What influence should this have on the number of viable cells at death of the susceptible host? One might predict that more viable cells would be required to kill an animal less capable of destroying the toxic pathogens than one whose defenses were intact. This may well be true but, when more than the number of cells found at death are administered as killed cells at the time of infection, the living pathogens still reach the same count when the animal dies. This can be seen in TABLE 8, and the result prevents any conclusions from being reached about the relative effectiveness of the defense mechanism. This is true even though the endotoxins of the heat-killed cells may be par-

TABLE 8

THE MEAN NUMBER OF VIABLE CELLS OF *SALMONELLA TYPHIMURIUM* FOUND PER GM. OF MOUSE AT THE TIME OF DEATH IN CONTROL MICE AND IN MICE GIVEN INTRAPERITONEALLY 0.25 AND 0.5 BILLION HEAT-KILLED PATHOGENS AT THE TIME OF INTRAPERITONEAL INFECTION

	Control mice infected intraperitoneally with 10^6 cells	Mice given 0.25×10^9 heat-killed cells at time of infection with 10^6 cells	Mice given 0.5×10^9 heat-killed cells at time of infection with 10^6 cells
Number of mice counted	13	9	12
Average number of pathogens per gm. of mouse carcass	53×10^6	49×10^6	45×10^6
Average survival time in hours	108	42	26

tially denatured and, consequently, not exactly equivalent to those released from the pathogens destroyed by body defenses.

Attention is directed to the fact that the number of bacteria per gm. of carcass in TABLES 8, 9, 10 is 3 to 5 times larger than the number shown in TABLE 4. The determinations presented in these former tables were made more than a year later than those in TABLE 4. While the culture was supposedly the same, there had been a slight reduction in virulence, as evidenced by increased survival times of infected animals. There is, however, insufficient basis for attributing these comparatively small differences in numbers of bacteria per gm. of mouse specifically to an altered virulence. Again, the total number of pathogens per mouse is obtained by multiplying the listed figures by 15, the average weight of the carcass. As shown in TABLE 8, there are significant differences in survival times in the 3 groups of animals.

Viable bacterial counts obtained from massively infected immune animals, compared to nonimmune control animals given a smaller infectious dose so that survival times will be roughly comparable, are recorded in TABLE 9. Here again no significant difference appears. From these data, therefore, one is forced to conclude that pathogen counts at the death of an animal tell nothing, within the limits of the method, of the relative number of bacteria destroyed by the animal's defense mechanisms.

Returning now to the question of the greater susceptibility of altitude-

TABLE 9

THE MEAN NUMBER OF VIABLE CELLS OF *SALMONELLA TYPHIMURIUM* FOUND PER GM. OF MOUSE AT THE TIME OF DEATH IN CONTROL MICE AND MICE IMMUNIZED PRIOR TO INFECTION

	Control mice infected intraperitoneally with 0.5×10^6 cells	Immune mice infected with 70×10^6 cells
Number of mice counted	13	14
Average number of pathogens per gm. of carcass	42×10^6	35×10^6
Average survival time in hours	128	94

TABLE 10

THE MEAN NUMBER OF VIABLE CELLS OF *SALMONELLA TYPHIMURIUM* FOUND PER GM. OF MOUSE AT THE TIME OF DEATH IN CONTROL MICE AND MICE EXPOSED TO SIMULATED ALTITUDE OF 20,000 FEET FOR 3 WEEKS PRIOR TO INFECTION

	Control mice infected intraperitoneally with 10^6 cells	Mice exposed to high altitude infected intraperitoneally with 10^6 cells
Number of mice	13	8
Average number of pathogens per gm. of mouse carcass	53×10^6	30×10^6
Average survival time in hours	108	62

exposed mice, it has been found that the number of viable bacteria at death is the same as it is for control mice. This may be seen in TABLE 10. While there is little more than one half the number of cells in altitude-exposed mice, as compared with the controls, the difference is not statistically significant according to the rank-order test of White (1952). Survival times, on the other hand, are significantly different at the 1 per cent level by the same test. Again it may be concluded that an essential distinction between the experimental and control groups is the time required for the "lethal" population of pathogens to be reached.

As a matter of curiosity, the possibility of a metabolic adjustment to the stress of simulated high altitude was investigated by analyzing several tissues of altitude-exposed animals for their citric-acid content. This specific compound was selected because of its position in the tricarboxylic-acid cycle and also because of the sensitivity and reliability of the method of Ettinger, Goldbaum, and Smith (1952). All assays were carried out on the pooled tissues of 3 mice. The results are given in TABLE 11. Notice that there is a progressive drop in citric-acid concentration up to the third week at altitude, and at that time all tissues except heart show approximately a 30 per cent decline in this intermediate. The heart tissue is 20 per cent below control values. These values remain unchanged after 4½ and 6 weeks in the decompression chambers.

TABLE 11

CITRIC-ACID CONTENT OF MOUSE TISSUES AT DIFFERENT TIMES OF EXPOSURE TO SIMULATED 20,000-FOOT ALTITUDE

Tissue	Citric-acid content of mouse tissues (μg./gm. wet wt.) in					
	Control mice	Mice kept at simulated altitude for				
		1 week	2 weeks	3 weeks	4½ weeks	6 weeks
Blood	36	30	25	22	24	22
Duodenum	104	80	76	75	70	71
Heart	63	51	53	49	50	50
Kidney	52	43	44	39	36	37
Liver	80	63	57	54	56	55
Spleen	96	71	66	66	66	68

TABLE 12

CITRIC-ACID CONTENT OF MOUSE TISSUES DURING DIFFERENT PERIODS OF RECOVERY FROM 3 WEEKS EXPOSURE TO SIMULATED ALTITUDE OF 20,000 FEET

Tissue	Citric-acid content of mouse tissues (μg./gm. wet wt.) in					
	Control mice	Mice kept at altitude for 3 weeks and then given a recovery period of				
		0 days	5 days	10 days	14 days	21 days
Blood	36	22	18	24	36	41
Duodenum	104	75	69	76	105	97
Heart	63	49	48	49	56	65
Kidney	52	39	36	39	47	50
Liver	80	54	57	80	84	80
Spleen	96	66	68	92	100	92

Following 3 weeks at altitude, the citric-acid concentration in the tissues has not returned to normal values after 5 and 10 days of recovery at normal atmospheric pressures. After 2 weeks, however, it is again normal, as seen in TABLE 12. In all instances no significant change in degree of hydration of the tissues accompanies exposure to altitude, so that the reduced citric-acid content appears to be the result of the establishment of a new "steady-state" concentration in the cells. There is at this time no information concerning the concentration of this intermediate in mice that are fully adapted to altitude for extensive periods of time, that is, for generations. On the basis of data now being accumulated in Peru at the laboratories of the Andean Institute of Biology, in Lima, approximately 50 feet above sea level, and in Morococha, approximately 14,900 feet above sea level, on guinea pigs native to the coastal plain and those that have lived for generations at high altitude, there is the suggestion that certain differences exist in citric-acid content of tissues but not necessarily in all tissues. If this is confirmed by additional data it will become important to know the nature of the citric-acid concentration in the tissues of animals during the period of adjustment to hypoxia.

A comparison of the susceptibility of mice exposed to altitude for different periods of time with that of normal mice, as seen in TABLE 13, makes it ap-

TABLE 13

MEAN SURVIVAL TIME IN HOURS OF DIFFERENT GROUPS OF MICE COMPARED TO CORRESPONDING CONTROLS

Experimental group	Number of mice	Survival time in hours	P value—experimental group vs. control
Control "A"	46	60	
Three weeks at altitude	40	42	>.01
Control "B"	65	75	
Six weeks at altitude	48	57	>.01
Control "C"	36	56	
Three weeks at altitude + 5 days recovery	36	39	>.01
Three weeks at altitude + 10 days recovery	36	42	.03

TABLE 14
MEAN SURVIVAL TIME IN HOURS OF DIFFERENT GROUPS OF MICE COMPARED TO CORRESPONDING CONTROLS

Experimental group	Number of mice	Survival time in hours	P value—experimental group vs. control
Control "D"	36	67	
Three weeks at altitude + 14 days recovery	36	69	<.70
Control "E"	44	177	
One week at altitutde	32	161	<.30
Two weeks at altitutde	31	159	<.20
Three weeks at altitude	38	141	.03
Three weeks at altitude + 14 days recovery	27	188	<.50

parent that susceptibility reaches a maximum at the time that the citric-acid content of tissues is minimum, and that it is still greater than normal while the citric acid is still depressed, that is, after 5 and 10 days of recovery at normal atmospheric pressures following 3 weeks in the decompression chambers. Note that different control animals were used for the different comparisons, since the number of mice employed prevented a single experiment from encompassing the different groups. The probabilities, as shown, were calculated by use of Student's *t* test. All differences between experimental and control animals are highly significant, including the one for those mice given 10 days of recovery.

The data of TABLE 14 demonstrate, however, that after 14 days of recovery, susceptibility, as revealed by survival time, is again normal. It is also seen that after 3 weeks at high altitude, but not after 1 or 2 weeks, susceptibility is increased. After 2 weeks of recovery from high altitude, however, resistance is normal. The last set of data are of interest because survival times are more than double those shown in the previous table. The severity of the infection does not appear to alter the results within the limits here observed.

Discussion

It would appear that disturbances artificially induced in the tricarboxylic-acid cycle of an animal greatly reduce survival time. This reduced survival time has not been correlated with a detectable change in those body defenses investigated. The only certain fact concerning this phenomenon, then, is the greatly accelerated rate at which the pathogens reach a relatively constant population at the time the animal dies.

Emphasis should be given to the fact that the change in host metabolism of mice exposed to high altitude, as evidenced by the altered concentration in tissue citric acid, is not necessarily linked causally with the greater susceptibility of the mice to infection. It is altogether possible that these events are independently correlated in time. At this stage of our understanding there has been observed merely the necessary agreement between changes in metabolism and in susceptibility for a cause and effect relationship to exist, but this is by no means sufficient evidence to establish such a relationship.

If it is to be assumed that altered metabolism leads to accumulation of intermediates that the bacteria can utilize for their nutrition, then the drop in citric

acid found in tissues of animals exposed to high altitude is in the wrong direction. It is obvious, however, that our information is limited to a single compound, namely, citric acid, and that other substances, about which we have no information, may accumulate.

Finally, mention should be made of the potential role of the adrenalcortical hormones in the altered response of the high altitude-stressed mice. There is now abundant literature showing that preinfection injections, particularly of cortisone, into experimental animals increases their susceptibility to a variety of pathogens (Kass *et al.*, 1953; Thomas, 1953). The underlying mechanism for these changes is not completely elucidated, but the reticuloendothelial system is clearly implicated. It is for this reason that emphasis is given to the fact that the clearance of thorotrast by liver, spleen, and lung of mice exposed to high altitude is normal. Moreover, spleen size, which becomes diminished under conditions of severe stress (Selye, 1950), is the same in our mice exposed to high altitude and normal mice. The specific proof, therefore, that we should attribute our observations to hormonal intervention, is lacking. Perhaps the explanation for the decreased citric-acid concentration in the tissues of mice exposed to high altitude is to be sought in the influence of cortical hormones on carbohydrate metabolism (Hoagland, 1947), but this must remain purely hypothetical until more specific evidence is available.

The work now in progress in Peru should contribute to our understanding of some of these relationships. Both short- and long-term effects of high altitude are being investigated. It is hoped that in time there will be a fuller knowledge of these problems.

Summary

This report is concerned with an analysis of possible mechanisms responsible for the shorter survival time of mice exposed to high altitude infected intraperitoneally with *Salmonella typhimurium* compared with that of ground-level control animals. The role of host metabolism in altering the course of bacterial disease is made evident by postinfection injections of sublethal amounts of inhibitors or intermediates of the tricarboxylic-acid cycle. Certain of these substances greatly increase susceptibility to a variety of pathogens. Since the average number of viable *Salmonella* at the death of an animal is the same in mice exposed to high altitude, mice with artificially modified metabolism and control mice, even though survival times vary over a wide range, it is concluded that the rate at which the "lethal" number of pathogens is reached is the primary variable. In both mice exposed to high altitude and in those injected with malonate following infection, no differences in those body defenses evaluated were found that would account for these observations. In mice exposed to high altitude, however, a decrease in citric-acid concentration of tissues reached a minimum value 30 per cent below normal after 3 week's exposure to hypoxia and remained unchanged after 6 weeks exposure. It returned to normal, following 3 weeks at altitude, after 2 week's recovery at ground-level pressures, but not after 5 or 10 days of recovery. Susceptibility to infection is greater in these animals at those times when citric-acid concen-

tration is depressed, and it is normal when the concentration of the intermediate is normal. This correlation in the timing of the metabolic adjustment and the altered susceptibility of hypoxic animals cannot be causally linked, however, at this time.

Acknowledgments

It is a pleasure to acknowledge the collaboration of the following former students in various aspects of the work contained in this report: Elenore Schewe, Penelope Merritt Ashmead, Jane E. Derbyshire, Marjorie Fair Read, Madeleine de Ropp, and Eva Schur. The technical assistance of Dorothy Smythe, Beverly Morris, Marylin Hill, and Edward K. Rose is also acknowledged with appreciation.

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BIOCHEMICAL STUDIES ON THE BACTERICIDAL POWER OF PHAGOCYTIC CELLS

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The vital role played by phagocytic cells in protecting animals from certain infectious diseases was first emphasized by Metchnikoff[1, 2] over 50 years ago. This concept has since been firmly established. Host resistance is, in some instances, clearly based primarily on the capacity of phagocytes to engulf and destroy bacterial invaders.

Surprisingly little is known concerning the intracellular biochemical conditions that kill parasites. The statement in most textbooks, namely, that microorganisms are "digested" in phagocytic cytoplasm, has no real meaning. It seems unlikely that they are killed by proteolytic enzymes, for living bacteria generally thrive in the presence of many ferments of this type.[3]

In this article I shall consider biochemical mechanisms that may account for the lethal effect of polymorphonuclear leukocytic cytoplasm on bacteria.

Organic Acids

Perhaps the simplest substances that may exert an antibacterial action inside phagocytes are the organic acids. Rous[4] first clearly showed that the reaction in phagocytic cytoplasm may be quite acid, and that the pH surrounding an engulfed particle appears to be near 4.5. Phagocytic cells possess a glycolytic metabolic apparatus and produce considerable quantities of lactic acid under both aerobic and anaerobic conditions.[5, 6] Presumably, accumulation of lactic acid accounts, at least in part, for the low pH often observed in these cells. A few years ago Dubos[7] demonstrated that lactic acid and several other organic acids have antibacterial properties when the reaction of the medium is below pH 6.0.

It is important to point out that a high degree of acidity may not be characteristic of normal leukocytic cytoplasm, but rather may develop as a consequence of engulfment of the foreign particle. This concept might well be termed "intracellular inflammation." In a sense, then, a principal role of phagocytes might be to sequester the parasites and to expose them to concentrated products of inflammation. Obviously host tissues in general cannot develop, for example, a high degree of acidity, for this would result in death of the animal. On the other hand, even if intraphagocytic acidity should, as may often be the case, lead to the death of the leukocyte, the host would survive since these cells are expendable.

The relative importance of acidity as a bactericidal mechanism of phagocytes remains somewhat speculative at present, since no accurate measurements have been made of pH, lactate, and other chemical conditions in intact cells.

Lysozyme

Until very recently the only bactericidal substance recognized to be present in polymorphonuclear leukocytes was lysozyme.[8, 9, 10] Lysozyme is a strongly

basic low molecular-weight protein that degrades enzymatically certain aminopolysaccharides. Exposure to lysozyme results in rapid lysis of bacteria whose cell wall is composed of this particular carbohydrate polymer. Although lysozyme is limited in its lytic action to a very few gram-positive cocci, it is possible that in certain circumstances it kills a wider variety of microorganisms without dissolving them. As an illustration of this possibility, it has been recently observed by Lack, Dubos, and myself[11] that many coagulase-negative staphylococci are killed but not lysed on exposure to lysozyme at a mildly acid pH, while neither of these agents alone produces a lethal effect. Coagulase-positive staphylococci, on the whole, are not affected by similar exposure. These findings are especially interesting in relation to previous studies by Rogers and Thompsett[12] showing that coagulase-negative staphylococci are rapidly killed within polymorphonuclear leukocytes, while coagulase-positive strains survive in this environment.

Phagocytin

That lysozyme and acid do not account for all of the antibacterial power of polymorphonuclear leukocytes is suggested by the fact that many microbes, coliform bacteria for instance, are not affected by lysozyme, even at an acid reaction, yet are known to be rapidly killed inside these cells. This fact led me to investigate[13, 14] the biochemical basis for the death of enteric bacilli within polymorphonuclear leukocytes.

Essential to the success of these experiments was the development of a technique whereby it is possible to collect from rabbits large numbers of intact polymorphonuclear leukocytes essentially free of debris and other cell types. When rabbit white cells so obtained are disrupted by any of a variety of physical methods and are then extracted with aqueous salt solution, the soluble portion manifests a striking bactericidal action on numerous gram-negative enteric bacilli. Susceptible to this lethal effect are strains of *Escherichia*, *Shigella*, *Salmonella*, *Proteus*, *Pyocyaneus*, and *Klebsiella*. Under the conditions thus far studied, several gram-positive bacteria, including staphylococci, streptococci, and mycobacteria, are unaffected by similar exposure. Although rapidly killed, coliform bacteria are not lysed by the rabbit leukocyte preparations.

The substance responsible for the lethal action of rabbit white-cell extracts on enteric bacilli appears to be, at least in part, a protein with general properties characteristic of a globulin. It is not dialyzable. It is inactivated by crystallized trypsin, and it is precipitated and may be quantitatively recovered from the fraction insoluble at 60 per cent saturation with ammonium sulfate.

This material is obviously different from lysozyme in both antibacterial activity and in precise chemical nature. Furthermore, there is abundant evidence indicating that leukocyte extracts are unrelated to properdin. For example, in contrast to the properdin system,[15] rabbit white-cell preparations exert bactericidal action in the absence of complement and magnesium ions. Louis Pillemer kindly examined some of the leukocyte extracts and was unable to demonstrate properdin or antiproperdin substances in them. Since this globulin from rabbit-polymorphonuclear phagocytes appears to differ from

previously characterized bactericidal substances from tissues, it has been called phagocytin, a name that connotes both its origin and its probable chemical nature.

Phagocytin is stable to temperatures as high as 65° C. for several hours, but on standing in the refrigerator for prolonged periods of time activity disappears. Thus a leukocyte extract stored at 4° C. for 3 weeks has only about one eighth the activity of the fresh extract. The reasons for the instability of phagocytin on storage are not yet clear, but they may well be related to the fact that it is inactivated, presumably by adsorption, on exposure to a wide variety of materials, including filter paper, porcelain candles, denatured proteins, and insoluble inorganic salts. Bactericidal activity cannot be eluted from any of these adsorbing materials.

As mentioned above, cytoplasm about a phagocyted particle is quite acid. It was therefore of special interest to find that the bactericidal activity of phagocytin is influenced by the reaction of the medium. The more acid the environment, the more marked is the bactericidal activity. In the range from pH 4.0 to pH 7.0, an increase in acidity of 1 pH unit produces a fivefold to tenfold enhancement of activity.

Although the mechanism by which phagocytin brings about the death of enteric bacilli is not yet established, some preliminary experiments have been done in this area. The number of bacteria present does not strikingly influence the activity of the leukocyte extracts. A thousandfold increase in the bacterial inoculum requires only a twofold to fourfold higher level of phagocytin to produce a given degree, for example, 90 per cent, of killing. The time-temperature relationships of the interaction between phagocytin and susceptible bacteria show that at 0 °C. practically no killing takes place, while at 38° C. the lethal action is rapid, being well advanced in 5 minutes and complete within 30 minutes. These observations are in keeping with an enzymatic reaction.

Extracts of polymorphonuclear leukocytes from mammals other than the rabbit have also been studied. Those of human and of guinea pig cells manifest bactericidal activity on coliform microorganisms, but this activity is much less than that of the rabbit preparations. Extracts of mouse and of rat leukocytes show no lethal effect on gram-negative enteric bacilli. It is possible, of course, that the nature of the bactericidal material or the conditions required for its action may differ among species of animals. On the other hand it might be pointed out that phagocytin is destroyed by proteolytic enzymes, and it has been previously shown that the rabbit polymorphonuclear leukocyte is unique in that it contains no demonstrable trypsinlike leukoprotease.[16] Thus perhaps phagocytin is obtained with ease from rabbit cells simply because they contain no enzyme that destroys it.

Summary

There would then appear to be at least 3 potentially bactericidal substances in polymorphonuclear leukocytic cytoplasm: acid, lysozyme, and phagocytin. Further work is required to establish which of these agents is of prime importance in determing the outcome of specific phagocyte-parasite encounters. The information at hand suggests strongly that various combinations of these cellu-

lar agents may exert a synergistic effect in destroying microorganisms. For example, the combination of lysozyme and acid kills certain staphylococci not susceptible to either alone, and the bactericidal activity of phagocytin is clearly enhanced at acid reactions.

It is admittedly true that many of the infectious diseases of most concern to mankind at present are due to microorganisms that survive intracellularly. A first step essential to an understanding of the resistance of these particular parasites is, of course, knowledge concerning the cytoplasmic conditions to which they are resistant. As presented above, some of the bactericidal factors in polymorphonuclear phagocytes have now been uncovered. No information is available, however, dealing with similar aspects of macrophage cytoplasm.

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PHAGOCYTOSIS-PROMOTING FACTOR OF PLASMA AND SERUM

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In the earlier part of this monograph there have been presented scholarly delineations of the various proteins, nutritional factors, hereditary factors, and possible biochemical factors that comprise the natural viricidal and bactericidal property of tissues and body fluids. I should like now to direct your attention to another line of defense in the maintenance of homeostasis: leukocytic phagocytosis, as mentioned by J. G. Hirsch elsewhere in this publication. This system requires the simultaneous presence of 3 component parts: the bacteria or particle to be ingested, living migratory cells to do the ingesting and, finally, extracellular protein factors capable of converting the phenomenon from an interesting laboratory demonstration to a rapidly consummated biologic phenomenon exerting significant effect upon host survival.

During the past 4 years our laboratory has been engaged in attempts to isolate and identify the proteins of plasma and serum responsible for this acceleration of phagocytosis. The presence of such phagocytosis-stimulating substances is not a new finding. In fact, the literature in this field dates back more than a half century. Unfortunately, however, this literature sometimes has been enmeshed in semantics: Should such factors be called opsonins? Should such factors be called alexin? Or, indeed, are such factors merely manifestations of complement activity? For purposes of simplification we have grouped all the protein factors that stimulate natural nonimmune phagocytosis under the descriptive term, phagocytosis-promotion factors or PPF. These factors will so be referred to in this report. Points at which such activity varies from the classic concepts of complement and opsonins will be amplified.

It should be noted that these protein factors are additive to the intrinsic ability of leukocytes to be ameboid and to ingest particles at a slow but measurable rate in even a protein-free medium. These PPF factors also are separate and distinct, we believe, from the specific phagocytic stimulation that occurs in a sensitized system of immune antibodies and appropriate bacterial strains. Finally, the PPF factors also *may* be independent of alterations in phagocytic rate that can be mediated through metabolic or hormonal influences acting upon the intracellular kinetics of the leukocyte.

What, then, are the PPF factors? We believe them to be the proteins that are concerned with the natural regulation of phagocytic rates within the intact animal. In this regard it should be noted that Y. Matoth, of the Hebrew Medical School, Jerusalem, Israel, while working with us a few years ago, demonstrated the presence of these stimulatory factors in the fetal-cord serum of newborn infants. Admittedly the phagocytic enhancement of these factors was less than that of the whole plasma or serum of the maternal circulation when added to a suspension of the same fetal leukocytes. Nevertheless, well-defined phagocytic stimulation was demonstrable under neonatal circumstances.

In the interest of brevity I shall delete detailed description of our assay methods. Three points only are of importance in this regard:

(1) All of the original source material from which our phagocytosis-enhancement factors have been isolated was derived from pools (or single units) of plasma or serum of healthy donors whose history was familiar to us and to whose freedom from active infections we could attest.

(2) The test particles used in our assays have been starch granules exclusively. Controlled studies on phagocytic rates and indices gave comparable results for *Amaranthus cruenthus* starch granules and different strains of staphylococci.

(3) The quantitation of biologic effect has been in terms of phagocytic index which, for this study, has been empirically defined as the total number of ingested particles per 100 cells per 1-hour incubation.

As a first step in identification of PPF, the effects of simple heating, freezing, aging, and coagulation were observed. It was found that the stimulatory effects of fresh serum and plasma were essentially the same. Fresh-frozen plasma showed a slightly greater phagocytosis-promotion factor, but this finding was not seen regularly. Heat treatment (+56° C. for 20 minutes) not only removed phagocytosis-promotion effect but resulted in inhibitory activity below that of the control specimen of plain cells and starch granules. A sample of plasma from the same donor collected 2 weeks prior to the experiment and stored at +4° C. showed a slight loss of stimulatory activity. This loss averaged 15 per cent in repeated experiments. This rate of decay could be accelerated by various physical and chemical means. Conversion of plasma to serum, for example, effected no immediate change in the total stimulatory activity, but resulted in a loss of 30 per cent after 2 weeks. Similar instability on storage was seen following freezing and thawing, or various chemical treatments.

As a second step in identification of PPF activity, fresh plasma was subjected to paper-strip electrophoresis. The technique was as follows: $\frac{1}{200}$ ml. of plasma or serum was placed on No. 1 Whatman analytical paper and submitted to electrophoresis for 16° at +4° C., pH 8.6, 150 volts. At the conclusion of each electrophoresis, a control strip was stained to localize the 4 or 5 major protein bands corresponding to gamma globulins, β-2 globulins, β-1 globulins, α-globulins, and albumin. The parallel unstained strip was then subdivided to correspond to each of these protein bands. The divided segments were promptly immersed in 0.4 ml. of standard Hank's buffer solution for 30 minutes. The segments were then squeezed with pinch forceps to elute the protein. They were then removed and discarded. Fresh white cells and starch granules were now added in amounts comparable to the standard assay, and the phagocytic activity was determined after 1-hour incubation at 37° C. Recovery of almost full phagocytic activity from a fragment of paper strip on which only $\frac{1}{200}$ ml. of starting material had been used emphasizes the extreme sensitivity of the system.

Two peaks of activity were noted consistently in all specimens of fresh plasma electrophoresis by this technique. One peak corresponded to the β-globulins

and the other peak to the rapidly migrating albumin fraction. This latter activity was not due to albumin, however, but rather to a closely associated α-1 globulin that could be isolated separately by the use of multiple 1-cm. cuts along the paper strip.

Having established the presence of 2 separate plasma proteins with phagocytosis-promotion activity, chemical studies were undertaken further to identify and purify both factors. Freshly collected resin plasma was subjected to Method XII of fractionation, both individually and in pools. This method, the last devised by the late Edwin J. Cohn of Harvard University, Cambridge, Mass., utilizes the interactions of proteins and heavy metals at normal pH. In each instance the original blood was collected, cooled, and rendered cell-free under optimal operating conditions in a Cohn centrifuge. The first step of Method XII consists of the addition of powered barium sulfate for removal of prothrombin and other coagulant proteins such as plasma thromboplastic component (PTC) and serum prothrombin conversion accelerator (SPCA). To our surprise, about one fourth of the phagocytic activity of plasma was removed by this single treatment. For a time we presumed that this part of the PPF-stimulatory activity might indeed be prothrombin itself, but recent work that is the subject of another report showed that the PPF factor that adsorbs on barium sulfate is a β-globulin separate and distinct from prothrombin. The factor can be recovered by elution of barium sulfate with citrate solution and is fairly stable on prolonged storage in the powdered state.

Effect of Zinc

Having established the interaction of the β-component of PPF with barium sulfate, zinc diglycinate was next added. This second step in Method XII forms the unstable globulins into a complex with zinc, leaving albumin in solution. The subsequent partition of the α-1 PPF factor was performed. Preliminary experiments suggested that the α-1 PPF was equally divided between the PGP (plasma globulin precipitate) paste and the supernatant stable plasma protein solution (SPPS) containing albumin and α-lipoprotein. If the conditions of fractionation were modified to include a preliminary dilution to 3 plasma volumes so as to avoid occlusion of SPPS in the globulin precipitation, however, all stimulatory activity was absent from the PGP and was present in the supernate. The PPF factor present in SPPS subsequently could be lyophilized and stored, as in the case of the β factor associated with barium. The separation of the α factor from albumin was possible by both paper-strip analysis and chemical means. Employing 1-cm. divisions of an electrophoresis strip of a redissolved SPPS, it was possible to show (FIGURE 1) that the maximal optical density (corresponding to the major protein component of this fraction, namely, albumin) was in the area of 10 to 12 cm., whereas the PPF activity was maximal in the closely associated areas of 8 and 13 cm. Similarly, the removal of albumin by fractionation of SPPS with 30 per cent ethanol left the supernate activity unimpeded. When redissolved, the albumin was not only free of phagocytosis-stimulatory activity but showed marked inhibitory effect below the control levels. This again corroborates the often

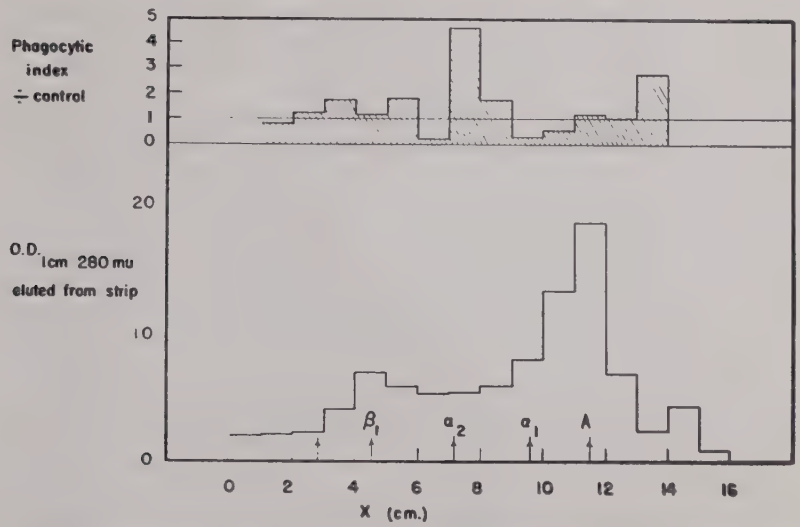

FIGURE 1. Electrophoretic distribution of factors from SPPS affecting phagocytic activity in isolated leukocytes.

neglected observation that albumin is the least desirable solution for *in vitro* cytologic study.

After the publication of Louis Pillemer's first work on properdin in 1954, our interest was aroused as to whether properdin showed PPF activities or, conversely, if PPF showed any properties similar to properdin. Accordingly, we studied the effect of zymosan treatment on the level of PPF in serum (TABLE 1). The standard technique of adsorption against 4 mg. of zymosan for 75 minutes at 17° C. caused an average decrease of 35 per cent in the phagocytic activity of whole serum. Subsequent heating at 37° C. for an added 75-minute period in the presence of magnesium ion did not further decrease phagocytosis promo-

TABLE 1

EFFECT OF PROPERDIN PREPARATION OF PHAGOCYTOSIS-PROMOTING FACTOR IN SERUM

Preparation	Test	Per cent phagocytic cells	Phagocytic index	Complement
Control	Control	30	175	−
Plain serum	Full PPF	71	570	+
Serum after cation resin	Effect removal Mg^{++} and Ca^{++}	69	439	+
Serum + 37° C. for 75′	Full complement Full Mg^{++} Full Ca^{++}	67	476	+
Serum + 4 mg. zymosan + 17° C. for 75′	Full complement No properdin	52	297	+
Serum + 4 mg. zymosan + 37° C. for 75′	No complement No properdin	61	323	−

tion. This phenomenon caused an initial impression that the 2 types of activity were somewhat similar, but subsequent analysis has not borne out this impression. Repeated assays of potent properdin preparations for phagocytosis-promotion activity have been consistently negative for any phagocytosis activity. This is in keeping with Pillemer's original findings. We have consistently been able to reproduce a loss of PPF activity by zymosan treatment of serum and, more recently, of plasma, however, so that our present thinking is to the effect that 1 of the PPF activities interacts with zymosan either in an irreversible complex or by some adsorption for which we have not as yet found the proper eluting agent. Of equal interest to this dissimilarity between properdin and PPF was the observation that subsequent heating to inactivate complement caused no further decrease in PPF activity. This observation would indicate a lack of activity of complement in phagocytic promotion as assayed in this system. Apparent corroboration of the absence of complementary activity in normal phagocytosis stimulation was obtained by paper-strip electrophoretic analysis of plasma with concurrent assay of the individual protein sections for PPF activity and for hemolytic complementary activity in a sheep-cell rabbit antisheep-cell system. These experiments, which suggest that complement does not have phagocytosis-stimulating activity, are in disagreement with a considerable body of literature dating back to 1905. Consequently, these data are advanced with trepidation for what value they might have. Suffice it to say that essentially all leukocyte studies in the past have been carried out in the presence of some complement, due to the known difficulty of washing leukocytes free of their nidus of plasma without significant damage and, of course, due to the impossibility of heat inactivation of such plasma in the presence of living cells.

Conclusion

A chemical and physical study of normal plasma and serum has shown 2 separate proteins that are stimulatory to phagocytosis by human leukocytes. These 2 factors, referred to in this paper as PPF, appear to be independent of complementary activity. One of the factors can be quantitatively adsorbed on barium sulfate, from which it can be eluted with citrate solution, lyopholized, and stored. Treatment of plasma or serum with zymosan for preparation of properdin reduces the PPF activity by about 35 per cent. No phagocytic activity has been found in purified preparations of properdin, and the 2 activities are apparently distinct.

SERUM BACTERICIDINS ACTIVE AGAINST GRAM-POSITIVE BACTERIA*

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Reports on the occurrence of bactericidal substances in serum and leukocytes date back to the early days of microbiology. In 1891 Pane[1] observed that the anthrax bacillus was arrested in its growth in rabbit serum even after heating the serum for 30 minutes at 55° C. His observations were confirmed by several other investigators who also demonstrated that sera from several mammalian species contained bactericidal activity for the same bacillus.[2-5] These investigations led to the point of view that certain sera contained bactericidal substances that were not dependent on complement and that appeared to be oriented more toward the gram-positive bacteria. Pettersson[6] referred to these substances as β lysins to distinguish them from Buchner's alexins or α lysins. Pettersson[7] believed that 2 components are involved in β-lysin action, β lysin and an "activating" substance. This belief was based on the observation that heat-inactivated serum could be made active again by adding a small amount of unheated serum.

Mackie and Finkelstein[8] and Mackie, Finkelstein, and Van Rooyen[9] studied the bactericidal activity of mammalian and avian sera on several strains of gram-positive bacteria. They failed to confirm Pettersson's observation, however, regarding the reactivation of heated sera by adding small amounts of normal serum. Tillett[10] reported on a bactericidin in human sera that is active against beta hemolytic streptococci. He noted that antistreptococcal activity was greatest in sera from patients in the acute phase of their disease, and he separated the *Streptococcus* bactericidal system into protein-free and protein-containing fractions that were effective only when mixed together. An agent active against *Staphylococcus aureus* was studied by Tobler and Pinner,[11] who found that pleural effusions from certain tuberculous patients inhibited the growth of their test organism. In a series of experiments on serum bactericidal activity against gram-positive bacteria of human and rabbit sera, Ostenfeld[12] reported that he was able to reactivate heated sera by adding small quantities of unheated sera.

More recently, Jacox[13] has studied a nonspecific substance bactericidal for *Bacillus subtilis* that appears in the sera of humans in the acute phase of a variety of diseases. The material was reported to require calcium ions for its activation, based on the observation that decalcifying agents inactivated the bactericidin and the addition of calcium restored activity. The calcium ions were presumably bound, since dialysis or treatment with a cation exchange resin failed to remove sufficient calcium from the serum to inactivate the system. This bactericidal agent was reported to produce lysis of the cells of *B. subtilis*. A bactericidal component active against *Bacillus anthracis* has

* This study was aided by a grant from the National Tuberculosis Association, New York, N. Y., made possible by a special bequest from the estate of Grace Velie Harris; and by a grant from the Virginia Tuberculosis Association, Richmond, Va.

been isolated by Bloom and Blake[14] from several tissues of various animal species. Electrophoretic and chemical analyses indicated that this substance is a basic polypeptide containing a large amount of lysine. Presumably, this component does not occur in normal serum, nor does it appear to produce lysis of sensitive organisms.

There exists a certain degree of unanimity among the investigators of gram-positive-serum bactericidins concerning the point that complement is not involved in bactericidal action. No clear picture exists, however, as to the number of systems or the broadness of the bactericidal activity with any given system. Furthermore, the problem of cofactors and activators taking part in gram-positive bactericidal systems remains unclarified. The lack of agreement in results is perhaps due to the multiplicity of bacterial strains employed by the numerous investigators in this area. The lack of concern about pH drifts in serum samples that lose carbon dioxide on standing could also produce spurious results. In addition, the possibility that some of the bacterial strains were sensitive to lysozyme would add additional confusion to the results. In this paper the bactericidal activity of selected mammalian sera is quantitated against *B. subtilis* and *Micrococcus pyogenes* var. *albus*. The bactericidins active against these 2 organisms in rabbit serum are studied in detail, and it is concluded that the bactericidins active against these 2 test organisms are very similar, if not identical.

Materials and Methods

Unless specified otherwise in the text, the following materials and methods were used throughout the investigation.

Organisms. The strain of *B. subtilis* employed was kindly supplied by R. F. Jacox. The coagulase-positive strain of *M. pyogenes* var. *aureus* and the coagulase-negative strain of *M. pyogenes* var. *albus* were obtained from the culture collection of the Department of Microbiology of the University of Virginia School of Medicine, Charlottesville, Va.

Quantitative bactericidin test. The bactericidin activity of the sera was determined by making doubled dilutions of the sera in saline in screw-capped tubes to give final volumes of 0.5 ml. The pH of the sera was stabilized at pH 7.2 by the addition of 0.1N HCl. Each tube was inoculated with 0.1 ml. of a saline suspension of the respective test organisms prepared from 3-hour nutrient broth (Difco*) cultures by washing once in saline and standardizing turbidimetrically with a Model-9 Coleman nephelometer to contain approximately 8 million organisms per ml. The inoculated tubes were incubated at 37° C. for 2 hours, at which time growth was readily visible in the high dilutions of serum. The end point was expressed as the highest dilution in which inhibition of growth of the test organisms was complete. Bactericidin activity was expressed in units, each unit being defined as the minimum amount of bactericidin that, when contained in a volume of 0.5 ml., will inhibit growth. For example, a serum capable of inhibiting growth in 0.5 ml. of a dilution of 1:8

* Difco Laboratories, Inc., Detroit, Mich.

would contain 16 units of bactericidin per ml. In all instances, bactericidin values were reproducible within the limits of a twofold dilution.

Quantitative lysozyme test. A lytic test described elsewhere[15] was employed for estimating the amount of lysozyme in the sera under study.

Animals. Adult Wistar rats, Hartley-strain guinea pigs, and New Zealand white rabbits furnished the sera for this study.

Results

Survey of bactericidin levels in mammalian sera. Sera were collected from some representative mammals and were quantitated for bactericidin activity against *B. subtilis* and *M. pyogenes* var. *albus*. Of the species tested, only the rabbit and the rat normally possess large amounts of bactericidins in their sera. The results (TABLE 1) indicate a parallel correlation between the activity of the respective sera and the 2 test organisms.

The remaining group of experiments was conducted with rabbit sera in order to study the possibility that a single bactericidin was active against both of the above organisms.

Effect of BCG in Falba and Bayol F on bactericidin levels. It has been reported[15] that rabbits receiving tubercle bacilli in mineral oil and Falba demonstrate a marked rise in their serum-lysozyme levels but no change in their *B. subtilis* bactericidin levels. The possible effect of this treatment on the "*ablus*"-"*subtilis*" bactericidin levels was investigated as follows:

Three albino rabbits weighing approximately 3 kg. were each given 80 mg. of cells of the BCG strain of *Mycobacterium tuberculosis* var. *bovis* in Bayol F and Falba subcutaneously in the inguinal region, and 80 mg. of BCG cells with the same adjuvant mixture intraperitoneally. The rabbits were bled from the marginal ear vein prior to the injection of tubercle bacilli and, at weekly intervals following the injection, for a period of 4 weeks. The sera were separated immediately after the clots had contracted and were stored in the frozen state until they were assayed for lysozyme, "*subtilis*," and "*albus*" bactericidin activity. The results obtained (TABLE 2) show that BCG in Bayol F and Falba produce a marked rise in lysozyme levels, but fail to affect the "*subtilis*" or the "*albus*" bactericidin levels. The results presented in TABLE 2 are typical of those obtained in the 3 animals in the experiment.

TABLE 1

BACTERICIDIN LEVELS IN SOME MAMMALIAN SERA AGAINST *BACILLUS SUBTILIS* AND *MICROCOCCUS PYOGENES* VAR. *ALBUS*

Source	*B. subtilis* (units per ml. of serum)*	*M. pyogenes* (units per ml. of serum)*
Rat	16–24	12–20
Rabbit	12–16	12–16
Guinea pig	less than 1	less than 1
Cow	less than 1	less than 1
Horse	1 (approx.)	1 (approx.)
Human (healthy)	1–2	1–2

* Unit is defined under materials and methods.

TABLE 2

THE EFFECT OF BCG IN BAYOL F AND FALBA ON THE SERUM LYSOZYME, "*SUBTILIS*," AND "*ALBUS*" BACTERICIDIN LEVELS IN RABBITS*

	Lysozyme μg./ml.	"*subtilis*" Bactericidin units/ml.	"*albus*" Bactericidin units/ml.
Prevaccination	0.6	16	12
1st postvacc. week	1.2	16	12
2nd postvacc. week	5.6	16	12
3rd postvacc. week	9.2	16	12
4th postvacc. week	11	16	12

* The results in this table were obtained from rabbit No. 230, and they are essentially identical to the results obtained in the remaining 2 rabbits.

Thermal stability of the bactericidins. The thermal stability of the bactericidal systems in rabbit serum against *B. subtilis* and *M. pyogenes* var. *albus* was determined by heating serum samples at 60° C. and testing for activity at 15-minute intervals up to 120 minutes. After a 30-minute heating period the serum gradually lost activity for both organisms and, at the 120-minute heating interval, all activity for the 2 test organisms was destroyed. Several attempts to reactivate such heat-inactivated sera with small amounts of normal unheated serum were unsuccessful.

Effect of sodium citrate on bactericidin activity. Jacox[13] reported that, when human sera possessing strong bactericidal properties for *B. subtilis* were treated by the addition of the sodium salts of citrate, oxalate, and phosphate, bactericidal activity was destroyed. He found that the addition of $CaCl_2$ reversed this inactivation, whereas $MgCl_2$ failed. In a previous communication from this laboratory[15] it was reported that the *B. subtilis* bactericidin in rabbit serum was also sensitive to sodium citrate.

This type of inactivation was studied by adding varying amounts of sodium citrate to samples of rabbit sera and subsequently testing such sera against the 2 test organisms. The results from this trial (TABLE 3) show that 6 units of either "*subtilis*" or "*albus*" bactericidin are completely inactivated in the presence of 0.045 M sodium citrate.

The mechanism of citrate inactivation of the bactericidins appears to involve factors other than calcium binding. Experiments studying the effect of ionic strength on bactericidin activity have revealed that the "*subtilis*" and "*albus*" bactericidal systems are very sensitive to increased ionic strength. In this regard it has been observed that the addition of calcium chloride or sodium chloride, to produce an added ionic strength of 0.1 or more, completely inactivates 6 units of either "*subtilis*" or "*albus*" bactericidal activity contained in a volume of 0.5 ml. Since sodium citrate is, for all practical purposes, completely ionized at pH 7.2, it was considered possible that sufficient sodium citrate existed in an ionized form to produce inactivation on this basis. Calculations of added ionic strength from TABLE 3 show that 0.045 M sodium citrate would be equal to 0.27 ionic strength, assuming that no un-ionized citric acid existed and that no reaction occurred between citrate ions and serum components. Actually, the amount of un-ionized citric acid present in this

Table 3
The Inactivation of "*subtilis*" and "*albus*" Bactericidins by Sodium Citrate

Materials (final volume 0.6 ml.)	Sodium citrate in mols				
	0.06	0.045	0.03	0.015	0.0075
Sodium citrate control in 1:20 serum	+	+	+	+	+
Six units "*subtilis*" bactericidin	+	+	−	−	−
Six units "*albus*" bactericidin	+	+	−	−	−

Symbols: + = growth; − = no growth.

system at pH 7.2 would be approximately 4.5×10^{-4} M which, for practical considerations, may be ignored.

The possibility that citrate inactivated the bactericidins, not by a process of decalcification but by increasing the ionic strength, was investigated as follows:

Serum preparations containing 6 units per ml. of the bactericidins were inactivated by the addition of sodium citrate to give 0.06 M final concentration. These mixtures were subjected to dialysis against 2 changes of distilled water for a period of 48 hours. Serum preparations without sodium citrate were dialyzed for an equal length of time to serve as dialysis controls. Controls on the citrate inactivation and undialyzed serum were also included. The preparations were double-diluted in the usual manner, inoculated with the 2 test organisms, and incubated for 2 hours at 37° C. It was found that dialysis treatment accomplished only a partial (25 to 50 per cent) reactivation of the citrate-inactivated sera. It is not known why a greater reversal of inactivation was not accomplished. These results would tend to rule out the possibility that calcium is a cofactor for this bactericidal system, although additional experiments are necessary to clarify this point.

Effect of bacterial products on bactericidin activity. Ekstedt and Nungester[16] recently reported that preparations of coagulase were capable of destroying the bactericidal activity of human serum against coagulase-negative strains of micrococci. They also observed that in most instances coagulase-positive micrococci were resistant to the human-serum bactericidal system. Accordingly, the rabbit-serum bactericidin system was investigated by testing its activity against a coagulase-positive strain of *M. pyogenes* var. *aureus*. This particular strain was completely resistant to the rabbit-serum bactericidin. The possible role of coagulase in destroying this bactericidin was investigated by growing a coagulase-negative strain and a coagulase-positive strain of *M. pyogenes* in meat infusion broth for 48 hours at 37° C. The cells were removed by centrifugation at 10,000 g for 30 minutes. It was found that 0.1 ml. of the culture supernatant fluid in which the coagulase-positive organism had grown was capable of coagulating 1 ml. of 1:2 human plasma within 60 minutes, whereas 1.0 ml. of the culture supernatant fluid in which the coagulase-negative organisms had grown failed to coagulate 1 ml. of human plasma during 24 hours of incubation.

TABLE 4
EFFECT OF COAGULASE-CONTAINING CULTURE SUPERNATE ON "*ALBUS* AND "*SUBTILIS*" BACTERICIDINS

	Dilution of serum				
	2	4	8	16	32
	B. subtilis				
Control-normal serum	−	−	−	+	+
Coagulase-neg. supernate	−	−	+	+	+
Coagulase-pos. supernate	−	−	+	+	+
	M. pyogenes var. *albus*				
Control-normal serum	−	−	−	+	+
Coagulase-neg. supernate	−	−	+	+	+
Coagulase-pos. supernate	−	−	+	+	+

Symbols: + = growth; − = no growth.

The respective culture supernatant fluids were incorporated into a rabbit serum known to contain full bactericidin activity. The culture supernate-serum mixtures (0.25 ml. culture supernate plus 0.25 ml. of serum dilutions) were incubated for 1 hour at 37° C. and inoculated with the test organisms *M. pyogenes* var. *albus* and *B. subtilis*. Approximately 25 per cent of the activity was lost by the addition of the culture supernates (TABLE 4). The coagulase-positive supernate, however, did not antagonize the bactericidin to any greater extent than the coagulase-negative supernate.

Another experiment was performed in which the coagulase-positive strain of *M. pyogenes* was allowed to grow in a high bactericidin serum for a period of 2 hours at 37° C., when the serum became visibly turbid. The cells were removed by centrifuging at 10,000 g and the supernatant serum was tested for bactericidin activity against the coagulase-negative strain of *M. pyogenes* and *B. subtilis*. The results in this experiment clearly indicated that the growth of the coagulase-positive strain of *M. pyogenes* did not result in an appreciable destruction of the "*subtilis*"-"*albus*" bactericidin activity.

Skarnes and Watson[17] reported that acidic polymers such as hyaluronic acid, glutamyl polypeptide, deoxyribonucleic acid, and ribonucleic acid inhibited lysozyme activity. They interpreted this antagonism to be due to direct combination of lysozyme and the acidic polymers through the formation of numerous salt linkages. In their test systems it was observed that 200 μg. of DNA inhibited 25 μg. of lysozyme, and 125 μg. of RNA inhibited 10 μg. of lysozyme.

The bactericidin systems in rabbit serum were tested for similar inhibition, employing DNA and RNA (nutritional biochemicals) as inhibitors. Experiments in which the bactericidin was kept constant and the concentrations of DNA and RNA were variable indicate that 6 units of either "*subtilis*" or "*albus*" bactericidin are completely antagonized by 625 μg. of RNA or 1250 μg. of DNA in a volume of 1.0 ml. These results lend support to the proposal that

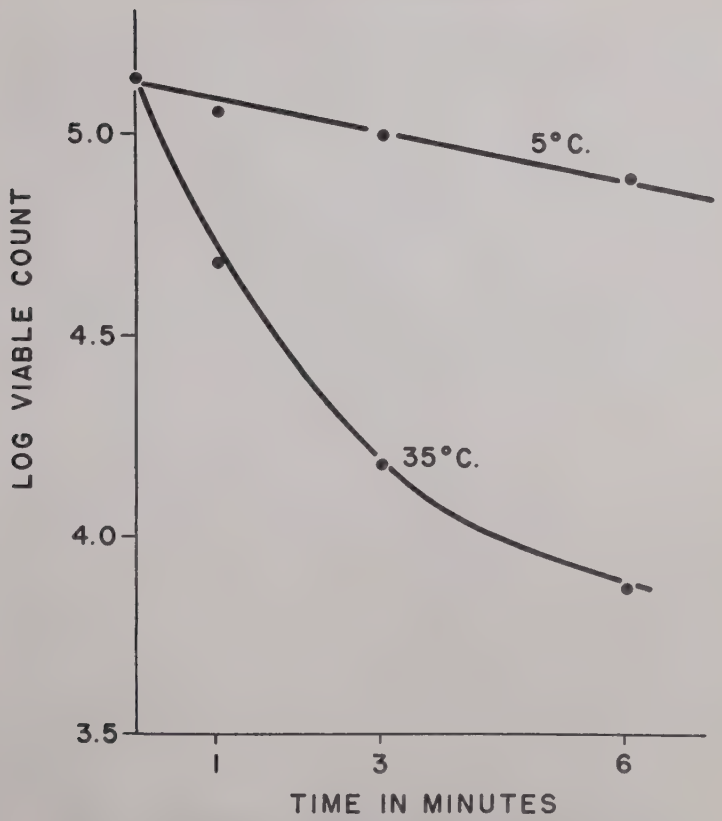

FIGURE 1. The lethal effect of 1:4 rabbit serum on *Bacillus subtilis* at 5° and 35° C.

the serum bactericidins are basic in nature. This proposal finds additional support in the observation that rabbit serum absorbed with bentonite[15] had no bactericidal activity for either *B. subtilis* or *M. pyogenes* var. *albus*.

In the experiments on properdin, Pillemer *et al.*[18] observed that zymosan absorbed properdin quantitatively from serum. Accordingly, following the procedure of these investigators, rabbit serum was treated with zymosan* and then tested for bactericidin activity. Approximately 50 to 75 per cent of the bactericidin activity against *M. pyogenes* and *B. subtilis* was removed by treating sera with zymosan. A preparation of egg-white lysozyme containing 100 μg. per ml. in saline was also treated with zymosan. In this case zymosan removed all detectable lysozyme, as measured with the lytic test against *Sarcina lutea*. These results suggest that zymosan is not a specific absorbent for properdin under the conditions employed in the above absorptions.

* Generously supplied by Standard Brands Inc., New York, N. Y.

Effect of temperature on the "subtilis" *bactericidin.* The lethal action of the "*subtilis*" bactericidin was studied quantitatively at 5° and 35° C. in an attempt to approximate the Q_{10} for the lethal reaction. In this experiment rabbit serum was diluted 1:4 with saline and was inoculated with approximately 8 million organisms in each ml. of diluted serum. Samples of the organism-serum mixture were removed at selected intervals, when they were diluted and plated in nutrient agar. Colony counts of viable units were made, and the results of the lethal effects at the 2 temperatures are depicted in FIGURE 1. The values plotted on the curves represent the averages obtained from triplicate counts. It should be noted that temperature affects the lethal action markedly. Dividing the slope at 35° by the slope at 5° and deriving the cube root of this figure indicates that the Q_{10} for the lethal reaction is between 2 and 3, which is in the range one might expect for an enzymatic reaction. The "*albus*" bactericidin was not studied with this method.

Discussion

Although the "*albus*"-"*subtilis*" bactericidal system in rabbit serum has not been isolated in purified form, all of the data obtained indicate that the active principles for *B. subtilis* and *M. pyogenes* var. *albus* are very similar, if not identical. They are similar with respect to (1) heat resistance, (2) sensitivity to ionic strength, (3) absorption with bentonite, (4) inactivation by sodium citrate, (5) quantitative distribution in mammalian sera, and are (6) antagonized by the same amount of DNA and RNA, (7) removed to the same degree by zymosan treatment, (8) not dialyzable, and (9) not increased in rabbits given tubercle bacilli and adjuvants.

This system does not appear to require any cofactors, and it is independent of the heat-labile components of complement. The data presented, however, do not unequivocally rule out cofactors in this system. It is lysozymelike in activity, with respect to the way it initiates a reaction that terminates with lysis of the susceptible cells. Experiments studying the lethal action at 2 different temperatures suggest that the bactericidin for *B. subtilis* behaves like an enzyme. This suggestion is further supported by the experiments on sensitivity to increases in ionic strength and to thermal inactivation.

The relationship of the bactericidin under study to the numerous bactericidins that have been reported is difficult to evaluate. In view of the loss of antistreptococcal activity observed by Tillett[10] when human serum was readjusted to pH 7.0, it seems likely that the factor he described is distinct from the factor studied in this laboratory. The failure to confirm Pettersson[7] and Ostenfeld's[12] observation that small amounts of normal serum reactivated heat-inactivated serum is unexplainable. In this regard, it should be mentioned that Mackie and Finkelstein[8] also failed to confirm this point.

The question also arises as to the possible relationship of the "*albus*"-"*subtilis*" bactericidal system to the properdin system recently discovered by Pillemer *et al.*[18] The experiments on heat stability appear to exclude properdin, since complement is reported to be an absolute requirement for activity of this latter system. No evidence was obtained that might suggest

that the "*albus*"-"*subtilis*" bactericidin system requires complement for activity. Furthermore, properdin occurs in fairly high levels in bovine and human serum, whereas the "*albus*"-"*subtilis*" system is extremely low in these species. Zymosan absorption did not prove to be a useful technique for distinguishing between the 2 systems, since the "*albus*"-"*subtilis*" system was largely, but not completely, removed by this treatment. Moreover, zymosan removed lysozyme "quantitatively" from saline-lysozyme preparations, which suggests that zymosan is not a specific absorbent for the properdin system.

Experiments with a coagulase-positive strain of *M. pyogenes* var. *aureus* indicate that coagulase is unable to destroy the "*albus*"-"*subtilis*" bactericidin system under the conditions described, and that coagulase does not function in an extracellular capacity, thus causing coagulase-positive strains to be resistant to this system. It does appear likely that the coagulase-positive strain of *M. pyogenes* var. *aureus* is resistant to the rabbit-serum bactericidin by virtue of either structural characteristics (capsule?) or a constitutive (metabolic?) resistance not dependent on extracellular substances.

Summary

The bactericidin activity against *B. subtilis* and *M. pyogenes* var. *albus* has been quantitated in the serum of the rat, rabbit, human, guinea pig, cow, and horse. Of these species only the rat and rabbit possess appreciable amounts of bactericidin.

The bactericidin in rabbit serum was studied in detail with respect to heat stability, citrate inactivation, effect of bacterial products on bactericidin activity, and effect of BCG in adjuvants on the serum levels in rabbits.

All of the data obtained in the present investigation support the idea that the bactericidin active against *B. subtilis* and *M. pyogenes* var. *albus* are very similar, if not identical.

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EFFECT OF LEVAN AND DEXTRAN ON THE INFLAMMATORY PROCESS

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Effects of complex bacterial polysaccharides in infection have been studied extensively.[1] On the other hand, knowledge of the ways in which nonionic simple polysaccharides affect defense response of the body is still sparse. In pathology, however, as in other branches of science, it may be preferable to proceed from a relatively simple case to the more complex situations rather than conversely. Detailed examination of the action of a simple polysaccharide on a host-pathogen situation might provide a background against which the operation of a complex polysaccharide might be elucidated more readily.

The levans and dextrans constitute families of simple nonionic microbial polysaccharides. They can be produced in quantity from sucrose and isolated in states of relatively high purity. They can be synthesized *in vitro* from sucrose by enzymes. There are means whereby their molecular weight may be regulated. Procedures are known for their measurement in tissues and body fluids. They are highly soluble in water and can be prepared as sterile solutions of good keeping quality. They are tolerated in high dosage by several species of laboratory animals. The levans and dextrans are thus a suitable choice for experimental studies of the mode of action of polysaccharides on disease.

Although levans and dextrans are "simple" in the sense that each is converted by hydrolysis to a single hexose, they are not truly simple in any wider sense. The dextrans are mostly branched, and the degree and manner of their branching vary with the bacterial strain forming the dextran and with the conditions of the synthesis.[2] Bacterial levans similarly are branched structures.[3, 4] As neutral polymers rich in hydrophilic groups, such substances might manifest a set of basically identical biological activities. The locations and disposal of these substances in the body, however, are expected to depend upon the size and surface of the polymer particles. Thus their biological activities can be expected to differ within limits both as to branching pattern and molecular weight.

The levan used in our experiments was isolated from a culture of *Aerobacter levanicum* growing on a sucrose-broth medium. The product contained less than 0.1 per cent of nitrogen (Kjeldahl). It was converted quantitatively to fructose on hydrolysis in a mild acid. The mass average molecular weight, as determined by sedimentation and diffusion measurements, was 17×10^6. A structural examination of the material has been carried out in our laboratory by D. S. Feingold. By methylation analysis it was shown that this levan molecule has a branched structure, the main linkage being of type C2 $\rightarrow$ 6 and the branch linkage exclusively of type C2 $\rightarrow$ 1. As in other bacterial levans whose structure has been examined previously, the degree of branching is approximately 0.1.[3, 4, 5] The native levan of *A. levanicum* will be designated hereinafter briefly as levan. To what extent the biological properties of this

substance are common to nonionic simple polysaccharides as a general class and more particularly to other levans remains to be determined. In a number of instances that will be noted, evidence has been obtained that a native dextran possesses properties that are quantitatively not unlike those of levan.

FIGURE 1 illustrates the course of transfer of levan from the peritoneal cavity of a mouse into the bloodstream. The amount of levan recoverable from the peritoneal cavity by washing decreased exponentially with time (peritoneal half life, about 2 hours). Most of the levan appeared in the blood. There it reached a peak at about 6 hours and declined more slowly, the level having fallen to 50 per cent of the peak value in about 22 hours.

Levan showed marked infecting-promoting activity (IPA) in mice infected peritoneally by *Salmonella typhi O 90.1*[6] The question arose whether direct contact between levan and *Salmonella* within the peritoneal cavity was involved in the observed IPA. Several ways of testing this were tried.[7] It was found that when levan was injected into the blood it did not appear in the peritoneal exudate in detectable amount during the observation period of 6 hours. Again it was found that L.D. $_{50}$ of *Salmonella* in the normal mouse was 10 times as high for bacteria injected intravenously as for bacteria injected intraperitoneally. Thus one can be reasonably sure that the lethal outcome of the intraperitoneal infection was determined extravascularly. Levan at different levels of dosage was administered intravenously to groups of mice that received graded doses of pathogen intraperitoneally. The course of the proliferation of *Salmonella* in the peritoneal cavity was followed and, at the same time, mice mortality rates were measured. Levan showed nearly equal IPA when administered intravenously and intraperitoneally. In normal mice,

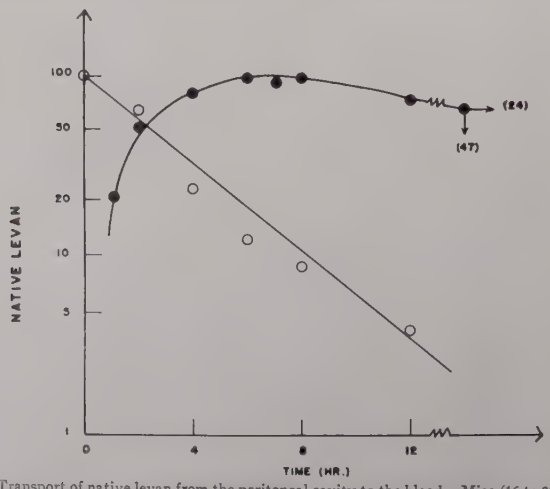

FIGURE 1. Transport of native levan from the peritoneal cavity to the blood. Mice (16 to 20 gm.) in groups of 3 to 9 animals each received 9 mg. of levan intraperitoneally. Each plotted point represents an arithmetic mean of a group. The abscissa shows the time (hours) after injection of levan, and the ordinate shows the concentration of levan in the blood (100 = 4.85 mg. levan/ml.) and the amount of levan in the peritoneal exudate (100 = original amount). The —•—•— curve represents the levan concentration in the blood, and the $\frac{\circ}{\circ}$ curve represents the amount in the peritoneal exudate.

TABLE 1

INFLUENCE OF LEVAN ON SYMPTOMS OF DERMAL INFECTION PRODUCED BY *M. PYOGENES*

Host		Polysaccharide			Total number of lesions examined	Response to pathogen	
Kind	No. of animals	Kind	Dose gm./kg. body weight	Route		Abscess formation*	Spreading dermonecrosis†
Guinea pig	5	none	—	—	24	+	0
Guinea pig	4	levan	0.5	intraperitoneal	15	0	+
Guinea pig	3	levan	0.5	intracardial	18	0	+
Guinea pig	2	dextran‡	0.5	intraperitoneal	7	0	+
Rabbit	20	none	—	—	40	+	0
Rabbit	15	levan	0.1	intravenous	20	0	+
Rabbit	3	levan	0.4	intraperitoneal	3	0	+

Levan was given vascularly concomitantly with the intradermal injection of *M. pyogenes*. Levan administered intraperitoneally was injected 2 hours prior to inoculation of skin by *M. pyogenes*.

* The symbol + designates regular evocation of visible erythema and edema at site of infection within 6 hours and formation of a localized purulent abscess at 20 hours. 0 designates the absence of these signs.

† The symbol + designates regular appearance of yellow-gray area of necrosis at about 6 hours with progressive lateral spread terminating at about 20 hours in a nonpurulent dermonecrotic lesion secondarily surrounded by an area of inflammation. 0 designates the absence of this response pattern.

‡ Native dextran prepared from a sucrose culture of *Leuconostoc mesenteroides*.

bacteria were cleared from the peritoneal cavity exponentially. In the levanized mice, the clearance mechanism failed to operate, and a rapid proliferation of the pathogen could be observed in their peritoneal cavities following a brief period of lag. Thus levan had altered the conditions prevailing in the peritoneal cavity even though levan itself had not entered the cavity. Obviously levan was affecting the host rather than the pathogen. These results suggested the possibility that the IPA of levan depends on an ability of the polymer to retard delivery of an antibacterial principle from the blood to the extravascular seat of infection.

The IPA of levan extended to tissue sites other than peritoneal cavity and comprised several species of host and pathogen. An infection produced by a strain of *Micrococcus pyogenes* was selected for more detailed study.[8, 9] The dermal lesion produced by this pathogen was one produced readily and uniformly in small laboratory animals and could be held under continuous observation from the outset and throughout the development. The influence of levan is shown by TABLE 1 and FIGURE 2. The effect of levan on the time course of development of the infection is presented by FIGURE 3. A striking difference between levanized and control animals is apparent already from the outset. In the normal host, the reddening and edema typical of acute inflammation became apparent macroscopically at about 1 hour with the subsequent intensification that culminates in the production of an abscess. In the levanized host, on the other hand, the infection remained free from external signs for 3 hours, a dermonecrotic reaction becoming suddenly visible in 4 hours, after which it showed a radial spread that continued steadily for 16 hours and then ceased suddenly.

These observations seem consistent with the interpretation that bacterial

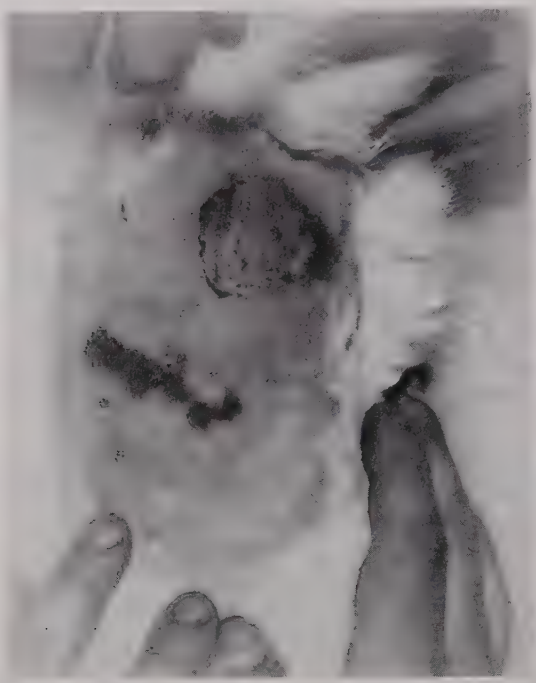

FIGURE 2. Dermonecrotic lesions evoked by *Micrococcus pyogenes* in the levanized rabbit. The rabbit received intravenously 200 mg. levan/kg. of body weight; 42-hour lesions. The top right-hand lesion, produced by the injection of the pathogen 5 hours before levan, shows dark hemorrhagic discoloration within a broad field of dermonecrosis. This is a characteristic feature in lesions of animals given levan only after the onset of evident inflammation. The bottom left-hand lesion, produced by the administration of the pathogen at the same time as levan, is a yellow-gray dermonecrosis with a narrow surrounding bond of hemorrhagic necrosis. In rabbits not given levan the response to the pathogen was a localized abscess.

invasion had proceeded for several hours in the levanized animals without opposing local concentration of defensive forces of the host upon the area of danger. In agreement with this result, it was found histologically that diapedesis failed to develop in levanized hosts at skin sites of infection by *M. pyogenes* for 10 hours, whereas diapedesis in nonlevanized hosts could already be observed 30 minutes after inoculation by the pathogen.[9]

The inhibitory effect of levan on diapedesis conceivably could have been mediated by the withdrawal of leukocytes from circulation, by an interference caused by penetration of the vascular wall by leukocytes, or by other means and their combinations, which we need not detail.[10] A prompt and large withdrawal of all leukocytes (notably neutrophiles) from the circulation was observed following injection of levan.[11] In the experiments shown in FIGURE 4, the leukopenic effect is seen to have remained in force for about 6 hours, after which the blood leukocyte count either reverted to normal or, transiently, to a hypernormal level. Native dextran, too, produced a marked but less durable leukopenic effect. Earlier workers[12] have reported the ability of high molecular dextran to lower the number of leukocytes in the circulation.

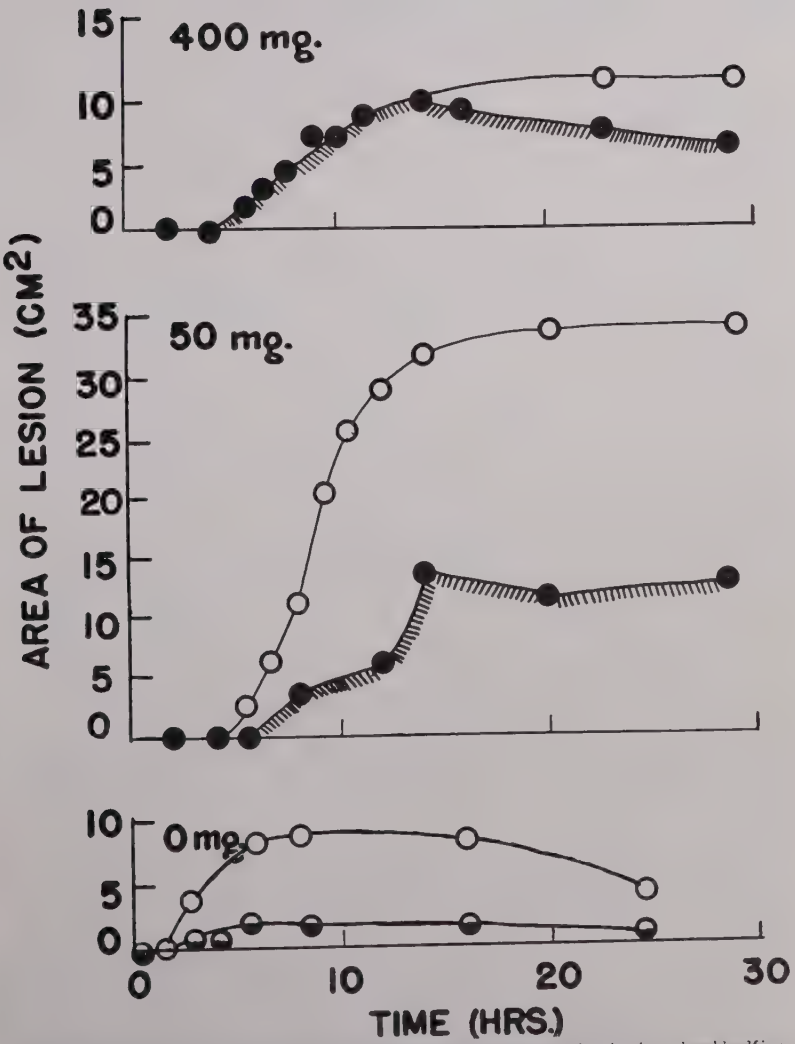

FIGURE 3. Influence of levan on the course of development of the dermal lesion that is produced by *Micrococcus pyogenes* in the rabbit. The rabbits (1.5 kg.) received respectively 0, 50, and 400 mg. of levan intravenously and were inoculated with *M. pyogenes* intradermally at the time of the levan injection. The open circles indicate the areas of the lesion comprising inflammatory involvement; the stippled areas, the areas of dermonecrosis; and the half-blocked circles, the area of purulent focus.

There is evidence that withdrawal of leukocytes from the circulation does not account fully for the failure of the levanized animal to manifest diapedesis. An opportunity to study this point was afforded in guinea pigs. M. Davies has observed that the leukopenic action of levan in this species is relatively

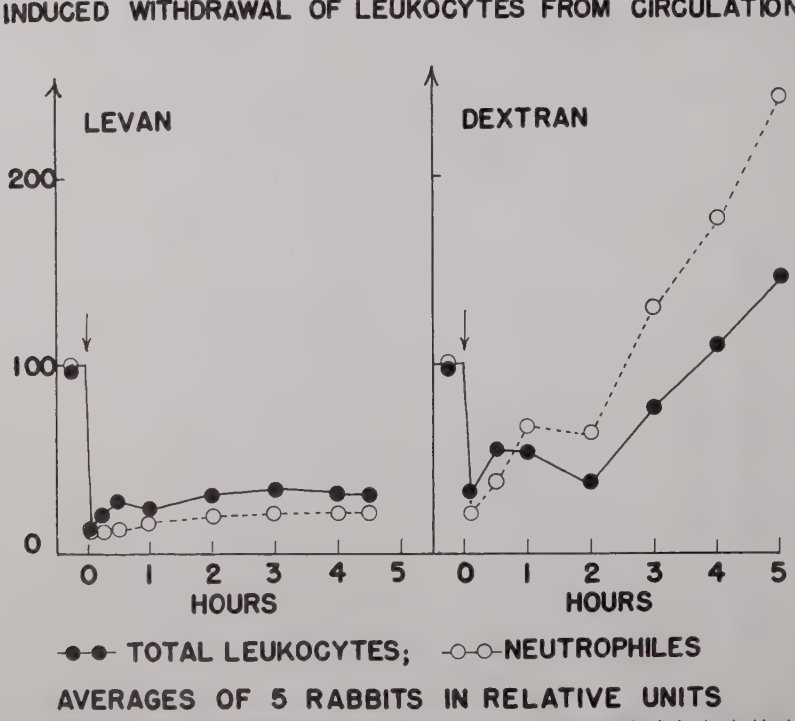

FIGURE 4. Effect of polymers injected intravenously on the number of leukocytes circulating in the blood stream. At the times indicated by the arrow (↓), the rabbits received the polymer (100 mg./kg. in 3-per cent solution) intravenously. The plotted values represent the means of the counts on 5 rabbits in each group. The levan was a native preparation of *A. levanicum*, m.w. 17×10^6 and the dextran was a native preparation, m.w about 10^6 (kindly provided by the Commercial Solvents Corporation, New York, N. Y).

short-lived. When levanized guinea pigs whose leukocyte count had been allowed to return approximately to normal after 4 hours were injected with *M. pyogenes* intradermally, a necrotic reaction, rather than abscess production, was elicited by the pathogen.

If it has been concluded correctly[14] that infiltration by leukocytes is necessary to the 'preparation' of an area of skin for a Shwartzman reaction, levan or dextran given intravenously prior to the injection of a preparing agent might be expected to prevent diapedesis and thus block skin 'preparation.' The same saccharides as colloidal agents might be expected to evoke a Shwartzman reaction at a prepared site. The experiment summarized in TABLE 2 shows that levan, like native dextran, is indeed both an efficient evoker of the Shwartzman reaction[12] and a suppressor of the skin-preparatory phase of the 'phenomenon.' A relatively weak inhibitory effect of middle-weight dextran and the apparent failure of clinical-size dextran to inhibit the phase of 'preparation' merit notice.[11]

In addition to inhibiting diapedesis, levan altered the inflammatory reaction

Table 2

Suppression of Skin-Preparatory Phase of Shwartzman Reaction by Native Levan and Native Dextran

Toxin dose (skin-preparing units)	Average diameter (mm.) of Shwartzman lesions in animals after pretreatment		
	Saline	Native dextran*	Native levan
60	14	1	2
30	11	1	0
6	7	0	0
3	5	0	0

Animals were pretreated by intravenous injection of polymer (100 mg./kg.) or saline, and they received graded doses of preparing agent (sterile filtrate of *Salmonella typhi* 0 901 homogenate) intradermally 30 minutes later. The evoking agent (levan 100 mg./kg.) was injected intravenously at 20 hrs.

* Dextran preparation, m.w. 5×10^6, kindly provided by Glaxo Laboratories, Ltd., Greenford, England.

by retarding transfer of certain colloidal plasma solutes through the capillary wall in inflamed areas.[8, 13] Both the leakage of globulins (including antibodies) and the administration of dyes through the capillary wall at sites of inflammation in the skin were found to be restricted by this agent. As is well known, animals given trypan blue dye systemically manifest a local blueing response in areas of injury in the skin. In rabbits and in some other species tested, a marked retardation of the blueing response, or even of its total suppression, was found to be accomplished by an intravenous administration of levan at the sites of inflammation evoked by physical agents (cold, heat, mechanical injury) or by chemicals (peptone and turpentine), provided always that the local injury was not overly extensive. To examine the effect of the levan on the leakage of antibody protein from the vascular bed to a tissue site of challenge, test systems were used in which a local inflammation and necrosis were induced by graded doses of toxin injected intradermally into animals passively immunized by intravenous injection of the homologous antitoxin. It was observed that the minimum amount of a *M. pyogenes* α-toxin producing visible dermonecrosis in a rabbit could be augmented roughly twentyfold by intravenous injection of a given quantity of a homologous antitoxin solution. The protective effect of the antitoxin was not obtained, however, in animals that had been given levan intravenously shortly before the antixoin. Levan given intravenously or intradermally to nonimmunized animals had no enhancing effect on the local dermonecrotising action of the toxin. It was also observed that levan in the blood did not alter the blood antitoxin titer. A titration of toxin by antitoxin in serum proceeded in the presence of levan as in its absence. These results indicated that levan had not bound antitoxin in the serum by a direct action, and that it was also probably without direct effect on cells of the skin. The findings, however, are consistent with the interpretation that levan opposed the protective action of circulating antibody against injury of skin by toxin because levan was about to lower the colloid permeability of the vascular bed towards the antibody in the area of the inflammation. A similar conclusion has also been indicated by experiments on a diphtheria toxin-antitoxin

Table 3

Action Loci and Acute Effects of Neutral High Polymers in the Circulation

Polymers:
Neutral polysaccharides: native levan and native dextran.
Observed effects:
(1) Enhancement of intraperitoneal proliferation of a pathogen (*S. typhi*).
(2) Failure of diapedesis in skin.
(3) Evocation of local Shwartzman reaction.
(4) Suppression of preparatory phase of the local Shwartzman reaction.
(5) Enhancement of dermonecrotic activity of a viable pathogen (*M. pyogenes*).
(6) Retarded blueing responses (trypan blue test).
(7) Enhancement of dermonecrotic activity of toxins in animals with passive immunity to the toxins.
(8) Binding of properdin.
(9) Withdrawal of leukocytes from circulation.

Targets of action of polymers:
(1) Endothelium
(2) Leukocyte
(3) Plasma

system in the guinea pig. Levan has been regarded on this basis as an agent that possesses endothelium-sealing activity (ESA).

The possibility further arises that levan can directly bind plasma solutes. This is particularly pertinent to systems in which properdin is involved. As has been shown by Pillemer and his colleagues[15] levan is a powerful adsorbent of properdin. As reported elsewhere in this monograph, these investigators have found that levan, like zymosan and lipopolysaccharides, lowers and then increases the blood properdin level. Thus it induces a primary phase of loss of resistance to infection and a subsequent phase of enhanced resistance.[16] Unlike the liposaccharides, however, levan is not a shocking agent, and it lacks acute toxicity.[14]

Native levan, like other efficient adsorbents of properdin, is a branched molecule of giant size. The high molecular weight of levan has been found to be an important factor in its biological activity. When the native polymer was converted by partial hydrolysis and appropriate fractionation to a levulan with such properties as are required in a plasma substitute, the product was found to be practically without either IPA or ESA.[7, 14]

In summing up, it is suggested that no single concept of the mechanism whereby polysaccharides affect the defensive mechanisms of the body is adequate. The loci of the action are clearly several: the capillary wall, the plasma, and the blood cells themselves (TABLE 3). The first of these is perhaps receiving less attention than it deserves. Studies of fundamental importance have been made in this field by Chambers and Zweifach[17] and Danielli and Stock[18]. In their work, the ability of polymeric substances, for example gelatin, administered in a perfusate to limit capillary permeability was first demonstrated and ascribed to the ability of such substances to form a coat upon the capillary wall. Levan and dextran form such capsular coats on bacteria forming these saccharides in a sucrose medium. Levan and dextran likewise tend to form such slimy coats upon a glass surface. Day[19] has demonstrated that dextran impacted into connective tissue lowers the rate of flow of water through such tissue. Pappenheimer[20] has shown that the rate at

which colloids can pass through capillary endothelium by restricted diffusion may be altered greatly by a minor change in the dimensions of an endothelial "pore." The hypothesis can thus be considered that levan administered systemically coats the capillary wall and thereby alters its permeability toward colloids and cells.

Acknowledgment

The work discussed in this paper was carried out in collaboration with M. Shilo, D. S. Feingold, B. Wolman, M. Wolman, and M. Davies.

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CONCLUDING REMARKS

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It would be impossible to do justice to this monograph by attempting a summary. I propose instead to make some observations on natural resistance and the strategy of its investigation.

In the first place, what do we mean by "natural resistance?" The solid, almost absolute, natural resistance of the hen to tetanus toxin, for example, is rather outside our field. This kind of resistance is an important biological fact, but we are more concerned with variations in partial resistance as displayed by a given species of animal; the kind of variation, for example, that Schneider dealt with. To talk in the abstract about resistance within a species, however, is meaningless. In our present state of knowledge questions about resistance are relevant only in terms of stated pathogens—resistance, for example, to a particular salmonella or to the malarial parasite.

As regards what we mean by "natural," I suggest that it is the resistance displayed by an animal that, as far as we know, has never experienced the stated pathogen, either as a pathogen or as a nonpathogenic parasite. This definition is meant to exclude specifically induced antibody immunity. Natural resistance in this sense, however, is not synonymous with nonantibody immunity, and it is not concerned only with nonspecific immunity. The newborn calf contains a natural agglutinin for *Trichomonas foetus* that can be distinguished from the antibody induced in later life by injection of *Trichomonas* antigens. Nevertheless, this agglutinin may well have something to do with the natural resistance of the young calf, and it is specific in that it does not agglutinate certain other trichomonads and even behaves differently with respect to 2 variants of 1 strain of *T. foetus*.[1] Again, the demonstration by Evans and Perkins [2] that intracerebral inoculation of dead *Hemophilus pertussis* in the mouse induces an early immunity to intracerebral challenge by the same organism, although it appears to be a nonantibody immunity, is nevertheless specific to the extent that the same early immunity is not induced by several other antigens, including *Hemophilus parapertussis*, *Hemophilus bronchisepticus*, *Escherichia coli*, *Chromobacterium prodigiosum*, and diphtheria and tetanus toxoids.

The articles included in this publication have successfully avoided antibody immunity, although to do so has been rather a struggle. Antibody has dominated the study of resistance for the last 70 years, sometimes to the detriment of research into natural resistance. There is a good and instructive reason for this dominance, one I suggest with due appreciation for all the subtle things that immunologists and immunochemists have done with antibodies. The reason is that antibodies are easy to work with because, carrying their own markers, they provide us with the guide and comfort of specificity. It is easy to set up the relevant controls. Antibodies have also dominated the study of defense reactions because they produce an enormous enhancement and acceleration of defenses (for example, phagocytosis) that are recognized

as nonspecific. Nonspecific reactions are thought of as the Cinderellas of immunology, awaiting the magic touch of antibody to transform them into something fit to go to the ball.

We must ask, however, whether nonspecific reactions are really so ineffective in the absence of antibody. They clearly keep the majority of animals in a state of equilibrium in which they do not succumb to the substantial microbial hazards of the environment. The majority of children who, in the Lübeck disaster, received a large dose of human tubercle bacilli by mouth clearly had a high natural resistance because only a small proportion of them died, and many did not manifest any serious disease at all. Moreover, if we consider the relatively large $L.D._{50}$ of most strains of pathogenic species that we can isolate from the environment, it is clear that our experimental animals, at any rate, have a substantial natural resistance. It may be objected that the high $L.D._{50}$'s indicate, instead of a high natural resistance, a low virulence of many of the strains that are tested, but this argument is based on the arbitrary assumption that natural resistance is, in fact, low. It is equally valid to assume a potentially high virulence and to postulate a high degree of natural resistance.[3]

Before leaving antibody, I should like to make a speculative point. Even if we work with the very early stages of infection in an animal that has never before experienced the pathogen, have we really avoided antibody immunity? The appearance of antibodies is usually timed from its exuberant production in amounts that spill over into the circulation of the animal—often a matter of days after the stimulus. With a large stimulus, according to Nunes,[4] pneumococcal antibodies can appear in the bloodstream within 5 hours of infection. Might they not appear, locally, at the site of infection even earlier? Antibodies are formed by certain cells of the lymphoid-macrophage system, a system characterized by a ready transformation of some of its constituent cells. If, in response to a local stimulus, a sessile histocyte can be transformed into an active phagocyte within approximately an hour,[5] why should other tissue cells not be capable of making some form of antibody response within the same short period after contact with the infecting agent?

What further dissection of natural resistance is possible? For convenience we can divide the stages of infection into: penetration; the primary lodgment of the pathogen, with or without large local spread; possibly a lymphatic lodgment; a reticuloendothelial lodgment; and, finally, generalization. This is an arbitrary sequence that does not occur in all cases, and the stages may often merge into one another. Nevertheless it is possible that some of the stages are functionally distinct in that they involve quite distinct and relatively independent defense reactions. We have found, for example, that substances that profoundly affect the defensive properties of the reticuloendothelial system, as tested by bacteremic infections, appear to have no effect upon local defenses in a tissue such as the skin. Again, Lurie's[6] work with pulmonary infections of rabbits by the tubercle bacillus suggests that resistance of the lung tissue to the primary lodgment can vary independently of the resistance of the body as a whole to generalized tuberculosis.

The dissection of these various stages in terms of natural resistance is not

easy. Penetration of what we might call the superficial constitutive defenses of the animal[7] is difficult to study with any precision. If we are to avoid the fallacies arising from the fact that the experimental injection or application of the pathogen bypasses certain natural defenses, we must undertake laborious and expensive studies in epidemiology, such as those of mouse salmonellosis by L. T. Webster in the United States and by W. W. C. Topley in Great Britain.

The chief methods of experimental attack, of which we have had many examples throughout these pages, have been applied to all stages of infection from the primary lodgment onward. These methods are (1) the effect of enhancers and depressors of infection, (2) the study of factors that change concomitantly with change in resistance, and (3) the analyses of defensive properties of isolated tissues and fluids.

The trouble with most enhancers is that they are blunderbusses in action. A great deal of the work on the action on infection of radiation sickness and shock tells us more about the radiation sickness and shock than about resistance. Nevertheless, the blunderbuss can be refined. In the work of Miller and Fine we have admirable examples of the analyses of such general effects into details that begin to tell us something about the way the resistance is altered in a given tissue. It is obvious that at a less blunderbuss level the pursuit of specific inhibitors of various kinds of tissue activity that are testable to some extent *in vitro*, offers a rewarding field of investigation. Berry's beginning with inhibitors of the tricarboxylic-acid-oxidation cycle is a case in point.

Even when we can pinpoint the site of action of an enhancing or a depressing factor, however, we are still up against the bugbear of nonspecificity. Until we define a cluster of characters that can substitute for the single specific test of the kind we have with, for example, an antibody, a cluster whereby we can identify an isolated factor in resistance with some precision, our conclusions are likely to be as nonspecific as the subject of our researches. In this connection, Pillemer was quite right in insisting that the functions of properdin could not properly be deduced from observation of, for instance, the bactericidal power of serum, or the change in resistance after the injection of zymosan or other microbial polysaccharides. Pillemer has provided a very definite set of characters for properdin, particularly in its relation to certain components of complement. Unless these characters have been elicited in a given investigation, properdin cannot safely be praised or damned for anything that is observed. The same principle must apply to all other factors isolated and described as significant in such studies.

As regards correlated changes, such as the parallel rise and fall of a serum factor and of a change in resistance, their interpretation requires caution. We have already been given some hints about the dangers of deducing causal relations from correlations, but it is also worth emphasizing how far mere association can lead us astray by recalling a demonstration made many years ago by Karl Pearson, one showing a high degree of correlation between the growth of the British Navy and the import of bananas.

I should add other general warnings. Some investigators have suggested unifying hypotheses of one sort or another underlying the phenomena of natural resistance. I confess that this suggestion rather frightens me. It is much easier to unify than to keep a multitude of disparate things in one's mind and biological material is very accommodating as a source of the kind of evidence we happen to be wanting. I recall Adami's remark, however, in his little classic on inflammation,[8] *à propos* of those who had worked with the rabbit cornea, that "the adherents to successive forms of inflammatory belief have found in experiments upon this simple tissue ample support for their particular creeds." Even at the risk of displeasing so strenuous an apriorist as Schneider, I shall insist that we must be what Robert Boyle called "sooty empirics," scrutinizing our experiments as descriptive biologists and then trying to knock sufficient (but *not* all-embracing) sense into the descriptions.

Even when we have a restricted hypothesis purged of all attempts at integration with the larger biological universe, we must avoid being forced out on a limb because of our experimental procedure. This can happen in 2 ways: our experiments may be too remote in kind from the natural process we investigate, and they may be too remote in degree. There is a good example of being caught out on a limb in the hypothesis of defense by lymphatic blockade that is based on studies of inflammation by substances like turpentine and pyogenic cocci in large doses. These irritants produce violent and often coagulative derangements of the tissues, but their effects are far removed from those characterizing the subtle march of invading bacteria in a natural infection, where the numbers penetrating the defenses in the first place are obviously small. Indeed, effective defenses at the primary lodgment appear to be independent of any thrombotic event whatsoever.

Finally, we should beware of accepting too readily explanations that fit conveniently and without obvious contradiction into general biological schemes. There is a cautionary tale in the history of histamine as a mediator of the inflammatory process. Here is an isolable and chemically defined substance, worked on for many years, whose effects mimic a number of basic inflammatory phenomena, and yet, with the exception of its action in a few allergies, mostly experimental, and as a gastric hormone, we still lack decisive proofs of any essential function for the large amount of histamine distributed throughout the animal body. The reputation of histamine as a natural mediator rests largely upon the fact that it *is* present and can be liberated in the body, thus encouraging the supposition that it therefore must do something useful.

The pitfalls awaiting the ready explainer are evident in the essay on the cow by a schoolboy who finds convincing reasons for the various features of the cow and, by his terse and authoritative style, makes them rather impressive. You will perhaps agree that his innocent philosophy does not differ very much from the thinking sometimes displayed by rather more sophisticated biologists.

"The Cow is a mamal, and it is tame. It has six sides, right, left, fore, back, an upper and below. At the back it has a tail on which hangs a brush. With this it sends the flies away, so that they will not fall into the milk. The

head is for the purpose of growing horns, and so that the mouth can be somewhere. The horns are to butt with. The mouth is to Moo with. Under the Cow hangs the milk. It is arranged for milking. When people milk the milk comes, and there is never an end to the supply. How the Cow does it, I have not yet realized, but it makes more and more. The Cow has a fine sense of smell. One can smell it far away. This is the reason for fresh air in the country."[9]

My summary has proved to be consistently critical, but I do not make the criticisms as a reaction to this monograph. On the contrary, I have tried to put into explicit form some of the critical principles implicit in the admirable articles included in these pages.

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